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Vol. XLI. *September, 1921.* *No. 3*

BIOLOGICAL BULLETIN

ANOTHER MALE COPEPOD OF THE GENUS SALMINCOLA FROM THE GILLS OF THE CHINOOK SALMON.

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The only male copepod known to exist in the genus *Salmincola* of the family Lernæopodidæ is that of *Salmincola edwardsii* (Olsson) Wilson, which parasitizes the brook trout *Salvelinus fontinalis* Mitchill of many of the states of the middle West and East. This male has been described and figured by the writer in the BIOLOGICAL BULLETIN, Vol. XXVII., for August, 1914. During the latter part of the summer of 1919, the author obtained large numbers of another species of *Salmincola*, namely, *Salmincola beani* Wilson, parasitic on the gills of the chinook salmon *Oncorhynchus tschawytscha* Walbaum of Green River, Auburn Washington, and a careful search amongst these parasites, resulted in the discovery of three mature males.

The size of these new male *Salmincola* is much larger than that of *Salmincola edwardsii* (compare Figs. 1 and 3 with 4). The former (Figs. 1 and 3) measure about 1.0 mm. in length and about 0.17 mm. in width in the region of the reproductive organs; whereas the latter (Fig. 4) are about 0.7 mm. long and 0.1 mm. wide in the region of the gonads. Otherwise their structure seems to be similar.

The organs which are of particular interest and importance in the male are the reproductive organs. These are paired structures, located laterally in the posterior region of the body (Fig. 1, *r*), between the digestive tract and the body wall. They consist essentially of the following three main parts: (1) the testes (Fig. 2, *t*), (2) the coiled vas deferens (Fig. 2, *v*), and (3) the

pear-shaped spermatophores (Fig. 2, *s*). The spermatozoa are manufactured in the testes, from which they are conducted into the vas deferens, and from here they are then conveyed into the spermatophores, where they are stored until the male is ready to fertilize the female. Located near the spermatophores, on the ventral side of the body are two openings, the ejaculatory pores (Fig. 2, *e*), through which the spermatophores may be extruded.

Fertilization in the Lernæopodidæ occurs shortly after the attachment of the free-living copepods to the host. In *Salmincola edwardsii* it takes place about two and one half to three weeks after attachment, and in the species under consideration, *Salmincola beani*, although the time of fertilization has not been accurately determined, it, in all probability, takes place after a similar lapse of time. When the copepods are ready for fertilization, the male is about one third or one fourth the size of the female.

Prior to fertilization the males and females hang side by side on the gills of the host, being attached by the so-called attachment filaments. In the male, the attachment filament remains long and tubular, with an enlarged bulla-like structure at one end which is firmly fastened in the flesh of the gill, while the opposite end is slightly enlarged and to it are attached the terminal claws of the male's second maxillæ. In the case of the female, the tubular portion of the attachment filament disintegrates shortly after the attachment of the female to the host, so that only the bulla-like portion remains for the attachment of the parasite.

In order to effect fertilization the male must seek out a female, release his hold on the gill, and then in some way become attached to the posterior margin of the female's body, in the region of the genital pores. The specific manner in which this is accomplished is as follows: When the male reaches maturity he undergoes circling movements. If he meets a female in the radius of his explorations, he grasps her body with the terminal claws of his free maxillipeds and at the same time releases his hold on the attachment filament. The male now creeps over the body of the female until he reaches the neighborhood of her genital

pores and here he attaches himself and remains until after fertilization. In Fig. 1 the male is seen attached to the middle of the body of the female, while in Figs. 3 and 4 the male is at the posterior margin of the female, in the vicinity of the genital pores ready to fertilize her.

Fertilization is accomplished by the male bending the posterior portion of his body towards the genital pores of the female. Then the male extrudes his two spermatophores through the ejaculatory pores, and by means of the second maxillæ he attaches these near the genital openings of the female. The spermatozoa soon wander through these openings and become stored in the spermatheca of the female. After the migration of all the spermatozoa, the spermatophores shrivel up and come to look like transparent, shell-like, yellowish spheres. Very shortly after fertilization the male dies, while the female lives on and develops a great many young, which are capable of carrying on the life-history of the species. In order to insure fertilization, it is by no means uncommon to find that many of the females have been fertilized by more than a single male.

BIBLIOGRAPHY.

Fasten, N.

'14 Fertilization in the Parasitic Copepod *Lernaeopoda edwardsii* Olsson. BIOL. BULL., Vol. 27, pp. 115–127.

Wilson, C. B.

'08 North American Parasitic Copepods: A List of Those found upon the Fishes of the Pacific Coast with Descriptions of New Genera and Species. Proc. U. S. Nat. Mus., Vol. 35, pp. 431–481.

'15 North American Parasitic Copepods belonging to the Lernaeopodidae, with a Revision of the entire Family. Proc. U. S. Nat. Mus., Vol. 47, pp. 565–729.

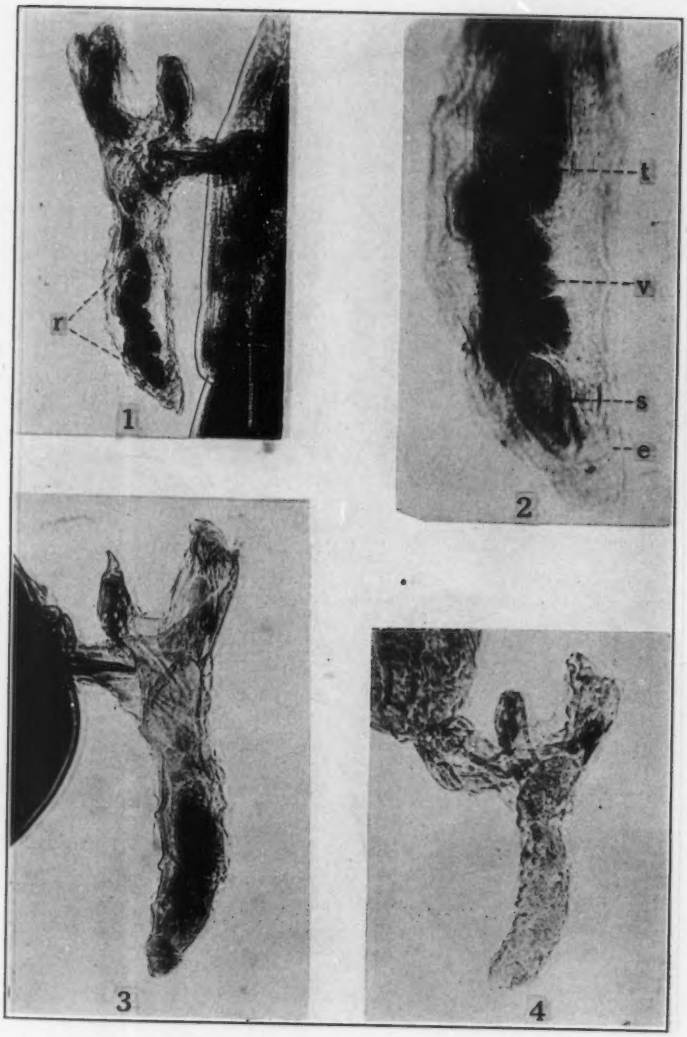
EXPLANATION OF PLATE I.

1. Male *Salmincola beani* Wilson, attached to middle of the abdomen of a mature female. The reproductive organs (*r*) can be distinctly seen. × 65.

2. An enlarged photograph of the reproductive organs of the male seen in Fig. 1. *t*, testis; *v*, vasa deferens; *s*, spermatophore; *e*, ejaculatory pore. × 183.

3. Male *Salmincola beani* Wilson in position near the genital pores of the female. × 65.

4. Male *Salmincola edwardsii* (Olsson) Wilson attached to genital region of female. × 65.



NATHAN FASTEN.

STUDIES OF FERTILIZATION, X.

The Effects of Copper Salts on the Fertilization Reaction in Arbacia and a Comparison of Mercury Effects.

FRANK R. LILLIE,

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1. Introduction.

Copper salts are known to have a profoundly injurious effect on many organisms even in high dilutions. They are also known to form compounds with ferments and proteins (on one side of the isoelectric point). It might therefore be expected in advance that they would have very definite effects in the delicate reactions of fertilization, which might be used advantageously in the analysis of these reactions, the precise character of which is still a matter of dispute.

The results reported were obtained in the summers of 1920 and 1921 at the Marine Biological Laboratory at Woods Hole. They have led me to the conclusion that the fertilization reactions in the sea-urchin are due primarily to activation of a ferment-like substance contained in the cortex of the egg which is presumably identical with the fertilizin of my earlier studies. The method of approach is quite new in fertilization studies, but has been previously used in ferment studies, as I discovered after the conclusion of my experiments. Certain points of comparison between inactivation of ferments by salts of heavy metals and inactivation of the fertilization reaction were found.

As soon as we adopt the conclusion that fertilization is essentially activation of a substance, contained in the cortex of the egg, by the spermatozoön (Lillie, 1914, 1919), the way is open for study of the properties of this substance by means of inhibitors of fertilization in general. If we were to determine the qualitative and quantitative range of such inhibitors we would be in possession of a list of properties of the postulated substance that

should enable us to classify it very closely. The present paper is a contribution to this program.

II. Methods.

The eggs of the sea-urchin, used in these experiments, should be washed in two or three changes of sea-water to rid them of blood or tissue secretions, and they should not be used for more than about three hours after washing. The quantities used should be small and constant for all experiments of a set; as a practical matter not more than two drops of eggs from the layer on the bottom of the container to 10 c.c. of sea-water. In more delicate experiments egg concentration should be expressed as number of eggs per c.c. of sea-water.

Sperm concentration may be expressed in terms of dilution of the dry sperm as I have done previously. For instance, one drop (0.1 c.c.) of dry sperm may be added to 25 c.c. of sea-water, and one drop (0.04 c.c.) of this suspension used to fertilize one drop of eggs in 7.5 c.c. sea-water. This may be expressed as one drop of 1:25:7.5 c.c. Such an insemination is abundant but not "heavy." It is equal to about 1.5 "units sperm concentration" of study VIII. Sperm concentration is given for all the experiments. A 1:25 sperm suspension should be prepared immediately before using and should not be used for more than about five minutes, as its fertilizing power diminishes rapidly. The main point is to make sure that the inseminations of a series are identical or comparable.

A solution of 1 per cent. by weight of c.p. copper chloride in distilled water was made; 0.1 per cent. in distilled water was made from this, and the various concentrations actually employed in the experiments were made immediately before each experiment by adding a definite number of drops of the 0.1 per cent. solution to 100 c.c. of sea-water, using a pipette graduated in hundredths of a c.c. on which the actual amount added was read off. The strength of copper chloride most commonly employed was 5 drops of 0.1 per cent. in 100 c.c. of sea-water. The readings of the 5 drops ranged around 0.19 c.c.; in round numbers this was calculated in as 0.2 c.c. and the amount of $CuCl_2$ in such a

solution is accordingly given as one part in 500,000. This is approximately 1/67,000 N, but on account of the alkalinity of the sea-water the effective concentration must be somewhat less.

In some early experiments a stock solution of copper sulphate in sea-water was used. The strength of this declined rapidly and it was used only in experiments 1 to 9. Its physiological effects are the same as copper chloride.

For each experiment there are two standard controls, viz.: (1) the *copper control*, *i.e.*, an identical insemination in copper chloride of the same concentration, and (2) *gamete control*, *i.e.*, an identical insemination in sea-water. Special experiments had in addition their special controls.

III. Experiments.

1. *The Phenomenon of Copper Inhibition. Relations between copper concentration and sperm concentration.*

The presence of one part of copper chloride in 500,000 parts of sea-water will completely inhibit fertilization of the eggs of *Arbacia*. Previous exposure of the gametes to the action of the copper chloride is not necessary for this result; if eggs and sperm are dropped simultaneously into the copper-containing sea-water, and mixed at once, no reaction occurs, though the sperm are as active as in normal sea-water. With most batches of eggs the inhibition is complete even when the amount of sperm used is several times what is necessary to fertilize 100 per cent. of the eggs in normal sea-water.

Though the eggs may be bombarded by hundreds of spermatozoa each, they do not usually give the first beginnings of the fertilization reaction; but, if any egg does so, fertilization is carried through to completion in the copper solution, and the egg segments. The inhibition is thus an "all or none" effect, as is shown more fully beyond.

The relations between copper concentration and sperm concentration are given in Table I. If the concentration of the copper is much greater than one part of copper chloride in 500,000 parts of sea-water, the inhibition may be regarded as complete for all sperm concentrations.

TABLE I.

RELATIONS BETWEEN COPPER CONCENTRATION AND SPERM CONCENTRATION. Time of exposure before fertilization = 0. Percentages are of eggs that segmented.

In these experiments the eggs were fertilized with definite concentrations of sperm (horizontal lines) in sea-water containing copper chloride to the amount of 1 part in 2,500,000, one part in 1,250,000, etc., to one part in 416,666 (vertical columns).

Sperm Concentration.	Sperm Concentration Units. See Study VIII.	1 / 2,500,000 $CuCl_2$	1 / 1,250,000 $CuCl_2$	1 / 833,333 $CuCl_2$	1 / 625,000 $CuCl_2$	1 / 500,000 $CuCl_2$	1 / 416,666 $CuCl_2$	Control No $CuCl_2$	Number of Experiment.
One drop 1 : 25 : 7.5 c.c.	1.5	50%	7%	0	0	0		100%	Exp. 35
Five drops 1 : 25 : 7.5 c.c.	7.5	100%	7%	50%	0	0		100%	Exp. 35
One drop 1 : 25 : 7.5 c.c.	1.5			5%	0	0	0	100%	Exp. 36
Five drops 1 : 25 : 7.5 c.c.	7.5			2%	1%	0	0	100%	Exp. 36
Two drops 1 : 5 : 7.5 c.c.	15			5%	1%	0	0	100%	Exp. 36
Five drops 1 : 5 : 7.5 c.c.	37.5			12%	2%	0	0	100%	Exp. 36
One drop 1 : 32 : 7.5 c.c.	1					0		85–90%	Exp. 14
Two drops 1 : 32 : 7.5 c.c.	2					0			Exp. 14
Four drops 1 : 32 : 7.5 c.c.	4					0			Exp. 14
Eight drops 1 : 32 : 7.5 c.c.	8					0			Exp. 14
Two drops 1 : 4 : 7.5 c.c.	16					0.2%		100%	Exp. 14
Four drops 1 : 4 : 7.5 c.c.	32					0.2%			Exp. 14
Eight drops 1 : 4 : 7.5 c.c.	64					0.5%			Exp. 14
Sixteen drops 1 : 4 : 7.5 c.c.	128					3%			Exp. 14

It will be noticed that inhibition is marked even at as low a concentration as one part of copper chloride in 2,500,000 parts of sea-water at a normal sperm concentration. Most of the experiments to be described were done with 1/500,000 copper chloride, at which concentration no eggs fertilize at normal sperm concentrations.

If much higher concentrations of sperm are used small percentages of eggs, varying somewhat in different experiments, may fertilize. (See last items of Table I.) There is thus a certain virtue in mass action of the sperm in the presence of this inhibitor of fertilization; this is somewhat difficult to understand, because only one spermatozoön penetrates usually. If we regard the spermatozoa, or some substance borne by them, and the copper as reacting with the same substance of the egg, it can be understood how the sperm substance could replace the copper in some cases when present in excess; or it may be possible that excess of sperm protects the eggs to a certain extent by combining with the copper and thus reducing the amount acting directly on the eggs.

2. *Reversibility of Copper Inhibition.*

Eggs that have been exposed to copper, whether in the presence of sperm or not, may be fertilized after return to sea-water provided that the exposure has not been long enough to injure their vitality too much. In other words, the inhibition by copper is reversible, and the phenomenon is to be regarded as one of inactivation of a substance, not of its destruction. In this respect, the phenomenon is precisely like the inactivation of an enzyme by mercury or copper salts, which is similarly reversible, and which has been shown to be due to a combining of the ions in question with constituents of the enzyme solution (v. Euler and Svanberg, 1920).

3. *Effect of Copper Chloride on Spermatozoa and Eggs Separately.*

A suspension of spermatozoa in 1/500,000 copper chloride has been tested up to 8 minutes exposure of the spermatozoa by fertilizing eggs in sea-water without any noticeable diminution in the fertilizing power of the sperm. The fertilizing power of a sperm suspension made in 1/25,000 copper chloride in sea-

water began to fall off rapidly after two minutes; after four minutes only 5 per cent. of the eggs fertilized.

Eggs, on the other hand, show an effect of exposure to 1/500,000 copper chloride from 10 seconds exposure on, if transferred and fertilized in sea-water. The effect is seen first in poor viability, then in increase of polyspermy (after 1 minute exposure), but they may form membranes even after 8 minutes exposure. If exposed to 1/25,000 copper, 50 per cent. will not fertilize at all after 1 minute. The eggs in short are much more sensitive to the copper than the sperm.

Unfertilized eggs left in 1/500,000 copper chloride in sea-water begin to show visible signs of injury after about two hours at (approximately) 20° C. The surface of the egg beneath the membrane first appears roughened, then by degrees a perivitelline space appears containing a fluid stained red by escaping pigment; a true cytolysis involving a "laking" effect due to destruction of the plasma membrane has occurred. At this time, except for the presence of pigment in the perivitelline fluid, the eggs look as though they were provided with fertilization membranes. Following this, cytoplasmic buds appear, the nucleus swells and the egg disintegrates very gradually.

4. *Effect of Copper Chloride on Fertilized Eggs.*

(*a*) If eggs are fertilized in normal sea-water and transferred to 1/500,000 copper chloride in sea-water two or more minutes after insemination, they continue their development for several hours, up to a late cleavage stage at least; but the copper acts as a slow poison, so that the eggs rarely reach a swimming stage. Eggs may segment in 1/250,000 and 1/125,000 copper chloride if transferred 5 or more minutes after insemination in sea-water; but their rate of death is naturally increasingly rapid. In general the length of life of fertilized eggs is about the same in any concentration used, whatever be the time of transfer before cleavage. Successive stages of fertilization after the first five minutes do not appear to vary notably in their sensitiveness to $CuCl_2$ within the range explored. The effect of $CuCl_2$ on the stages of fertilization after the first few minutes is thus a progressive poisoning varying in rate with concentration.

There is thus an extraordinary contrast between fertilized and unfertilized eggs in their sensitiveness to copper; it is obvious that copper has an effect that belongs to a separate order of sensitiveness on the early stages of fertilization as compared with later stages.

(*b*) The question thus arises as to the time limits of the early inhibiting copper effect. A number of experiments were performed to test this point, two of which are presented in Tables II. and III. In these experiments the eggs were inseminated in sea-water and then transferred at intervals to the copper solution. Samples of the eggs shown in Table III. were transferred back to sea-water after fourteen minutes in the copper solution to test their viability.

TABLE II.

(EXP. 12.) EFFECTS OF COPPER CHLORIDE ON EGGS INSEMINATED IN SEA-WATER. A very heavy insemination was used in this experiment: two drops of 2 : 5 : 7.5 c.c.

	Transfers to ½/500,000 $CuCl_2$.	Per Cent. Segmented.	Remarks.
(*a*)	4 seconds after insemination.....	10%+	Irregular cleavage.
(*b*)	6 seconds after insemination.....	10%+	Irregular cleavage.
(*c*)	10 seconds after insemination.....	80–90%	Irregular cleavage.
(*d*)	20 seconds after insemination.....	80–90%	Irregular cleavage.
(*e*)	30 seconds after insemination.....	100%	Irregular cleavage.
(*f*)	60 seconds after insemination.....	100%	Irregular cleavage.
(*g*)	Control in sea-water.....	100%	Normal.
(*h*)	Control inseminated in the Cu solution.....	1%	Irregular cleavage.

Here we must note two results particularly:

(1) Eggs that have begun fertilization reaction before transfer to the copper solution go through to cleavage, while those that have not begun it are instantaneously inhibited. Transfers made even four seconds after insemination (Table II.) include a considerable proportion of eggs (varying with sperm concentration in various experiments from 5 to 25 per cent.) which complete the fertilization and segment. The percentage rises to normal with increase of time before transfer.

(2) Eggs transferred from normal insemination to the copper chloride within the first two minutes do not give a complete

membrane reaction. The membranes of such eggs are "narrow," or better, the perivitelline space separating egg and membrane is narrow, often extremely so, down to the point of invisibility. The viability of such eggs is bad even if they are returned to sea-water again within a few minutes (Table III.). Indeed, the viability forms an ascending series to normal from 10 seconds up to 5 minutes. In other words, the fertilization reaction exhibits gradation in intensity according to the point of time at which the copper begins to operate, reaching normal at about two minutes with the best lots of eggs, and viability is proportional to fertilization intensity. There is no recovery from an initial subnormal reaction.

TABLE III.

(EXP. 37.) EFFECTS OF COPPER CHLORIDE ON EGGS INSEMINATED IN SEA-WATER. Insemination, one drop of 1 : 5 : 5. Eggs replaced in sea-water after 14 minutes' exposure to $CuCl_2$. This experiment came at the end of the season when the eggs were not in first class condition.

Transfers to 1/500,000 $CuCl_2$.	Per Cent. Segmented.	Remarks.
(*a*) 10 seconds after insemination.....	15%	Membranes "narrow."
(*b*) 20 seconds after insemination.....	30%	Viability bad.
(*c*) 25 seconds after insemination.....	30%	
(*d*) 40 seconds after insemination.....	50%+	
(*e*) 60 seconds after insemination.....	60%	Membranes "narrow."
(*f*) 90 seconds after insemination.....	70%	Viability better.
(*g*) 2 minutes after insemination.....	80%	Viability still better. Not equal to control.
(*h*) 5 minutes after insemination.....	80%+	
(*i*) 10 minutes after insemination.....	90%	Equal to control.

The period of time involved in these subnormal effects is that of membrane formation or of cortical discharge.

We have, thus, three distinguishable effects of copper chloride at successive stages of fertilization:

1. To inhibit activation if present at insemination.

2. To reduce the intensity of the cortical discharge, if its action begins within the first two minutes, proportionally to the time of its operation.

3. A slow cumulative injurious effect thereafter which belongs to a different order of events from 1 and 2, and which does not become obvious until late cleavage.

The data then give rise to the hypothesis that there is an activable substance present in the cortex of the unfertilized egg for which copper possesses special affinity. Copper thus prevents the inception of activation when present before insemination; during membrane formation it causes a quantitative reduction of activation. Thereafter, this substance having been consumed, the specific copper effects are absent.

Copper affects primarily the *activation* of this substance, and presumably not its *operation*, or at least to a very much less extent. The quantitative effects of the first two minutes can be understood in terms of the respective amounts activated and unactivated at the time of exposure to copper.

Using our earlier term *fertilizin* for the activable substance of the egg we may now attempt to form a picture of what actually happens in the copper chloride sea-water.

When a given lot of eggs is inseminated normally there is an interval of time taken up by the meeting of the spermatozoa and the eggs. The length of this interval will naturally vary statistically for any given insemination, and will tend to be inversely proportional on the average to sperm and egg concentration. Fertilization proper begins after the actual agglutination of the spermatozoön to the surface of the egg, following a latent period of variable duration. The activation of the fertilizin is then begun and the egg becomes sterile to other spermatozoa. As Just ('19) expresses it, a "wave of negativity" sweeps over the surface of the egg from the point of attack of the successful spermatozoön. Now the rate of this wave is sufficiently rapid to prevent polyspermy even at high sperm concentration where the eggs are in their best condition; the wave must therefore be completed very rapidly. As a result of activation the egg engulfs the spermatozoön within one minute or less, and the fertilization membrane elevates beginning at the point of entrance of the spermatozoön (cf. Just). Thus the events are (1) agglutination of the spermatozoön to the egg, (2) latent period, (3) activation and sterilization, (4) penetration of the spermatozoön, (5) membrane formation.

It does not necessarily follow that all of the fertilizin is acti-

vated in the first wave of negativity; this wave may be entirely superficial and activation may thence extend into deeper layers of the cortex. When, therefore, eggs are inseminated in sea-water and then transferred within the first few seconds to the copper sea-water there will be a certain proportion in which the initial reaction has begun, and this proportion will be larger according as the sperm concentration is higher, as actual experiments show. The copper will, however, instantaneously check the activation of more fertilizin so that the eggs must operate in the copper sea-water with that portion already activated. Transfers at later stages will be successively less affected by the copper, as successively greater amounts of the fertilizin will have been activated before transfer, until all is in operation. When this point is reached, at membrane elevation, the other events of fertilization proceed as well in the copper sea-water as in normal sea-water.

If this form of interpretation is correct, we can perhaps see a little farther into the fertilization reaction. If activation of fertilizin follows immediately after agglutination of the spermatozoön to the egg, we would expect in the case of a heavy insemination that activation would have begun in all of the eggs within a few seconds, say 5 to 10, after insemination, because it is practically certain that in that space of time all eggs will have agglutinated spermatozoa. However, we notice in both of the tables and especially in Table III. that the proportion of eggs that segment does not suddenly rise to normal in a ten-second exposure. In some cases the rise is relatively sudden, as in Table II., in other cases relatively slow, as in Table III., and this corresponds to the physiological condition of the eggs as shown by the promptness of their behavior in normal fertilization. In other words, it is necessary to postulate a latent period after agglutination of the spermatozoön before activation begins, which varies with the physiological condition of the egg. This fact could be brought out only by use of a reagent like copper that instantaneously checks activation. When the wave of activation is begun it spreads flash-like over the surface, though this rate varies also with physiological condition as shown by the relation of the latter to

polyspermy. It is not necessarily the first spermatozoön that agglutinates that effects the initial discharge, but this will depend on physiological condition of the spermatozoön, and also perhaps on local differences of physiological condition on the surface of the egg.

5. *The Effect of Copper on Agglutination of Spermatozoa.*

The objection might be raised that copper inhibits by preventing agglutination of the spermatozoön to the egg, and not in the later activation of the fertilizin. The effect of copper on the agglutination reaction of the spermatozoa was therefore studied: Egg-water (of 1,600 agglutinating units) diluted 100 times or more with 1/500,000 copper chloride in sea-water has the same agglutinating effect on a sperm suspension in the same copper solution as when no copper is present either in the egg-water or sperm suspension. Agglutination will occur even in the presence of one part of copper chloride to 25,000 parts of sea-water, though more slowly. Copper chloride in the concentrations employed in the fertilization experiments has no noticeable effect on rate or duration of the agglutinating reaction.

The inhibiting action of copper must then occur after the agglutination of the spermatozoön to the egg, as was assumed in the preceding discussion.

6. *Protective Action of Egg-Water Against Copper.*

If copper chloride inhibits by combining with the fertilizin of the egg, then egg-water which contains fertilizin should protect against the inhibition of fertilization by deviating the copper from the fertilizin in the egg to that in the egg-water. Now it is known that egg-water contains a sperm-activating, a sperm-aggregating and a sperm-agglutinating substance (Lillie, '14, '19), and I have identified the sperm-agglutinating substance with fertilizin. These considerations led to experiments on the protective action of egg-water on the copper inhibition, in which definite positive results were obtained.

The egg-water is obtained by placing eggs in sea-water which receives their secretions. The strength of the egg-water depends on egg-concentration primarily, and also on time to a certain extent; it may be measured, as far as the sperm-agglutinating

constituent of the egg-water is concerned, by finding the greatest dilution at which it will cause visible agglutination in a sperm suspension. If the proportion of eggs to sea-water is about 1 to 4 in bulk, the egg-water from fresh eggs will usually stand dilution to about 1/1,600, and would therefore be accordingly rated as 1,600 units agglutinating strength. Other constituents of the egg-water are diluted at the same time naturally, but there is no present means of measuring these.

In a preliminary experiment (No. 21) eggs were identically inseminated (one drop of 1:25:7.5 c.c.) in (*a*) normal sea-water, (*b*) in sea-water to which one part of copper chloride in 500,000 parts of sea-water had been added, (*c*) in egg-water of 1,600 agglutinating units to which also one part of copper chloride in 500,000 parts of egg-water had been added. In (*a*) 100 per cent. of the eggs segmented, in (*b*) none segmented, in (*c*) 68.5 per cent. segmented. The egg-water was thus shown to protect against the copper chloride.

More elaborate experiments were then set up, one of which is given in Table IV. Here it is shown that egg-water alone

TABLE IV.

(EXP. 23.2.) IDENTICAL INSEMINATIONS IN THE FLUIDS (*a*) TO (*k*). (One drop of 1 : 25 : 7.5 c.c.)

	Per Cent. of Eggs Segmented.
(*a*) 1/500,000 Copper egg-water of 400 agglutinating units	50%
(*b*) 1/500,000 Copper egg-water of 200 agglutinating units	50%
(*c*) 1/500,000 Copper egg-water of 80 agglutinating units	50%
(*d*) 1/500,000 Copper egg-water of 40 agglutinating units	15%
(*e*) 1/500,000 Copper egg-water of 20 agglutinating units	10%
(*f*) 1/500,000 Copper egg-water of 10 agglutinating units	<0.1%
(*g*) 1/500,000 Copper egg-water of 7 agglutinating units	<0.1%
(*h*) 1/500,000 Copper egg-water of 5 agglutinating units	0
(*i*) Sea-water alone. Control 1	80%
(*j*) 1/500,000 Copper sea-water. Control 2	0
(*k*) Egg-water alone (400 units). Control 3	50%

reduces the percentage of cleavage from 80 per cent. (i, control 1) to 50 per cent. (k, control 3); that 1/500,000 copper chloride in sea-water completely inhibits fertilization (j, control 2), and that egg-water protects completely against this concentration of copper chloride down to 80 units (a, b, and c), and that its protective action at lesser concentrations falls off to zero (d, e, f, g, h).

7. *Effects of H and OH Ions on Copper Inhibition.*

Two series of experiments (Nos. 24 and 25) were run to see if increase in H or OH ions protected against the inhibitory action of copper chloride. As might have been expected, increase in acidity of the sea-water had no favorable effect. On the alkaline side some protective action is found between about 1/2,500 N NaOH and 1/666 N NaOH by volume. In the best case about 33 per cent. of the eggs segmented when 1/666 N NaOH was added to 1/500,000 copper chloride sea-water. The membranes were, however, very narrow, and the cells tended to separate in cleavage owing to this fact. The appearance of the eggs was similar to eggs segmenting in a copper solution too weak to produce complete inhibition. The effect was no doubt due to precipitation of some of the $CuCl_2$ as $Cu(OH)_2$, but the dissociation of the copper salts was still sufficient to inhibit very greatly.

8. *The Protective Action of Gum Arabic and Gelatin Against Copper.*

Egg-water stands by no means alone in protecting against the inhibiting action of copper on fertilization. The same result may be obtained with either gum arabic or gelatin, and presumably with other colloids and proteins.

The protective action of gum arabic begins at 0.2 per cent. and becomes complete at 0.8 per cent. (see Table V.). As the gum arabic has no deleterious action of its own it furnishes complete protection at the proper concentration, as the table shows.

TABLE V.

(EXP. 32.) PROTECTIVE ACTION OF GUM ARABIC. Identical inseminations in the fluids (*a*) to (*e*) and their controls. (One drop of 1 : 25 : 7.5 c.c.)

One Part of $CuCl_2$ to 500,000 Parts of	Per Cent. of Eggs Segmented.	Same Solutions Without $CuCl_2$ (Controls).	Per Cent. Segmented.
(*a*) 0.2% gum arabic in s.w.....	10%	(*a*) 0.2% gum arabic in s.w.....	100%
(*b*) 0.4% gum arabic in s.w.....	55%	(*b*) 0.4% gum arabic in s.w.....	100%
(*c*) 0.8% gum arabic in s.w.. ..	100%	(*c*) 0.8% gum arabic in s.w.....	100%
(*d*) 1.6% gum arabic in s.w.....	100%	(*d*) 1.6% gum arabic in s.w.....	100%
(*e*) Sea-water (copper control)..	0	Sea-water only.....	100%

The protective action of gelatin begins at 0.008 per cent. and becomes complete at 0.064 per cent. (see Table VI.).

TABLE VI.

PROTECTIVE ACTION OF GELATIN (EXP. 34). Identical inseminations in the fluids (*a*) to (*j*) and their controls. (One drop of 1 : 25 : 7.5 c.c.)

One Part of $CuCl_2$ to 500,000 Parts of	Per Cent. Segmented.	Same Solutions Without Copper (Control).	Per Cent. Segmented.
(*a*) Sea-water (control)	0	(*a*) Sea-water (control)	100%
(*b*) 0.0001% gelatin in s.w.	0	(*b*) 0.0001% gelatin in s.w.	90%
(*c*) 0.001% gelatin in s.w.	0	(*c*) 0.001% gelatin in s.w.	90%
(*d*) 0.002% gelatin in s.w.	0	(*d*) 0.002% gelatin in s.w.	100%
(*e*) 0.004% gelatin in s.w.	<1%	(*e*) 0.004% gelatin in s.w.	100%
(*f*) 0.008% gelatin in s.w.	20%	(*f*) 0.008% gelatin in s.w.	100%
(*g*) 0.016% gelatin in s.w.	50%	(*g*) 0.016% gelatin in s.w.	100%
(*h*) 0.032% gelatin in s.w.	90%+	(*h*) 0.032% gelatin in s.w.	100%
(*i*) 0.064% gelatin in s.w.	100%	(*i*) 0.064% gelatin in s.w.	100%
(*j*) 0.1% gelatin in s.w.	100%	(*j*) 0.1% gelatin in s.w.	100%

Thus both gum arabic and gelatin protect against the inhibiting effect of copper. Presumably any non-injurious substance that would form a non-dissociable compound with copper, thus removing it from the sphere of action of fertilization, would similarly protect. The question then arises whether the protective action of egg-water is a special instance of the general colloid (gum arabic) effect, or of the protein (gelatin) effect. I think we may say definitely that the action of egg-water is not a general protein effect, for even the strongest egg-water does not give any certain protein reaction. It is moreover practically certain that diluted egg-water which protects does not have a colloid content equivalent to the protective minimum of the gum arabic solution. There is something in the egg-water that does not give protein reaction, but with probably an equal or greater avidity for copper. There is also something in the cortex of the egg itself with a similar affinity for copper, viz.: the activable substance. It is reasonable to suppose that these two things are identical.

It is an interesting fact that egg-water also possesses a deviating effect on the inhibiting action of species-blood in fertilization which is not a general colloid effect nor yet a general protein effect (Lillie, 1914). As egg-water protects against two such different forms of inhibitor, it is reasonable to suppose it is able to do so by possessing the same substance as that on which the

inhibitors work in the egg. No other hypothesis possesses the simplicity of this one, which means that the activating substance of the egg (fertilizin) is present in the egg-water.

9. *The Effect of Copper on Activation by Butyric Acid.*

If copper chloride inhibits fertilization by combining with the fertilizin of the egg it should also inhibit activation of the egg by parthenogenetic agents as, *e.g.*, butyric acid. This consequence of the theory was found to be true. If unfertilized eggs of *Arbacia* are placed in 50 c.c. sea-water + 2 c.c. *N*/10 butyric acid, and transfers are made to 1/500,000 copper sea-water and to sea-water for control, after 30 seconds and 45 seconds, it is found that in the sea-water good to fair membranes form on practically all of the eggs in about two minutes; but in the 1/500,000 copper sea-water no membranes form and the eggs appear entirely unchanged for at least twenty minutes. Then a gradual cytolysis begins to set in, entirely similar to the cytolysis that occurs in copper sea-water without previous exposure to butyric acid after two to three hours. The butyric acid has hastened the appearance of the copper cytolysis; but the copper has entirely inhibited the typical membrane-forming reaction.

Copper present with the butyric acid does not, however, inhibit the membrane formation after transfer to sea-water. In this reciprocal experiment one part of copper chloride is added to 500,000 parts of the butyric acid solution, and the eggs receive the normal exposure. When transferred to sea-water, membranes form just as though the copper had not been present. Thus, whatever the pre-activation effect of the butyric acid may be, copper does not inhibit it, but operates only to prevent the activation of the fertilizin.

Similarly in fertilization the preliminary effect of the spermatozoön in preparing the fertilizin for discharge ("latent period"; see p. 141) is not presumed to be affected by the copper but only the actual discharge (*i.e.*, activation) of the fertilizin.

10. *A Comparison of the Effects of Mercuric Chloride.*

It will be desirable to compare the effects of the salts of other heavy metals on fertilization, and some experiments have been begun along this line. However, in this paper, mercury alone will be considered.

The effect of mercuric chloride ($HgCl_2$) on fertilization is pronounced, but it is very different from that of copper chloride: the initial stages of fertilization are relatively little affected, and the susceptibility increases as fertilization progresses; fertilized eggs show the effects much more rapidly than unfertilized. Mercury also suppresses the movements of the spermatozoa at great dilution, and offers in this way another contrast to copper.

Table VII. records an experiment in which eggs were inseminated in various dilutions of $HgCl_2$ in sea-water, between one part in 1,250,000 and one part in 15,625. Most of the eggs form fertilization membranes in the four lowest concentrations. But the eggs do not segment, except at the lowest concentration tested, and then only 20 per cent. irregularly. The fertilized eggs cytolyze more rapidly than the unfertilized.

TABLE VII

EXPERIMENT 31A. EGGS INSEMINATED IN SERIES OF $HgCl_2$ SOLUTIONS IN SEA-WATER. (One drop of 1 : 25 : 7.5 c.c.)

$HgCl_2$ in Sea-water.	Membranes.	Cleavage.	Cytolysis Begins.	Action on Sperm.
1. 1/1,250,000 . . .	100% good	20% irreg.		Faint movement only in 3 min.
2. 1/625,000.	100% good	None		Same
3. 1/312,500.	90%+ good	None	68 minutes	Same
4. 1/156,250.	90%+ good	None	Bad in 1 hour	Paralyzed, 3 min.
5. 1/125,000.	20% good 80% none	None	Fert. eggs cytolyze in 20 minutes	Paralyzed in few seconds
6. 1/62,500.	15% good 85% none	None	Same as above	
7. 1/31,250.	2% 98% none	None		
8. 1/15,625.	None	None		Paralyzed instantly
9. Sea-water control.	100% good	100%		

It was also observed that unfertilized eggs form membranes in solution 5 (Table VII.) beginning at about five minutes. When transferred to sea-water such eggs undergo changes similar to eggs treated with butyric acid.

Mercury thus exhibits an extraordinary contrast to copper: in certain comparable concentrations it paralyzes sperm, copper does not; it produces membrane formation alone, and favors it in fertilization, copper inhibits membrane formation; it suppresses cleavage and cytolyzes fertilized eggs rapidly; copper is neutral during the same stages.

The differences in the actions of copper and mercury respectively on fertilization may be explained by differences in intensity of action on the early and the later stages of fertilization respectively. In the case of mercuric chloride I have found that the inhibiting action in fertilization (which occurs at higher concentrations than in the case of copper, as for instance in solutions 5 and 6 of Table VII.) is reversible, like copper. Specifically, eggs that remain unfertilized in these solutions may be fertilized after return to sea-water, if the exposure is not too long (about 20 minutes). Presumably, therefore, mercury acts like copper in the initial stages of fertilization, though a higher concentration is required; but in the later stages mercury acts deleteriously in much lower concentrations than copper; one part of $HgCl_2$ in 625,000 parts of sea-water will completely inhibit cleavage of fertilized eggs, whereas it requires about one part of $CuCl_2$ in 62,500 parts of sea-water to produce comparable effects.

The comparison may be tabulated as follows according to the stages of fertilization:

1. Agglutination of the spermatozoön to the egg.
2. Latent period.

   Copper intervenes, Hg not, or Cu>Hg.
3. Combination of sperm receptors with fertilizin.
4. Activation = combination of fertilizin with egg receptors.
5. Sterilization = "wave of negativity."
6. Spermatozoön enters the egg.
7. Membrane formation.
8. *Hg>Cu in later events.*

It is well known that the salts of heavy metals have a powerful "poisonous" effect on enzymes. McGuigan (1904) determined that for diastase the order of poisonous effects (complete inhibi-

tion) is $N/100{,}000$ silver (nitrate), $N/33{,}333$ gold (chloride), $N/30{,}000$ mercury (chloride), $N/8{,}333$ copper (chloride). v. Euler and Svanberg (1920) studied the effects of heavy metals on the inversion of cane sugar by saccharase, and determined among many other things that mercury has about one thousand times the poisonous effect of copper; the metallic ions in question enter into combination with some constituent of the enzyme solution.

The order of poisonous effects of mercury and copper on the events of fertilization following membrane formation is thus the same as that of the poisonous action of these metals on enzymes. The concentrations also are of comparable magnitude (cf. McGuigan's data above). v. Euler and Svanberg found the range for $HgCl_2$ between complete inhibition of the ferment and no effect to lie between 1 part in 116,600 and 1 part in 1,166,000 of the solution (compare Table VII. of this paper). There is thus a rather surprisingly close agreement in the effective concentrations; so that it is reasonable to conclude that the effect of mercury and copper on fertilization following membrane formation may be due to enzyme poisoning.

The inhibiting effect of mercury and copper on the initial stages of the fertilization reaction is in the inverse order, and does not, therefore, correspond so well to the enzyme analogy. The results emphasize the strong contrast between the initial and the subsequent events of the fertilization process, which is found also in the phenomena of specificity (Lillie, 1919, 1921), and in other phenomena of fertilization.

LITERATURE.

v. Euler, H., und Svanberg, Olaf

'20 Ueber Giftwirkungen bei Enzymreaktionen. I., Inaktivierung der Saccharase durch Schwermetalle. Fermentforschung, III. Jahrgang, pp. 330–393. II., Inaktivierung der Saccharase durch organische Stoffe, *ib.*, IV. Jahrg., pp. 29–63. III., Ueber den Einfluss von Kupffersulfat auf die Autolyse der Hefe, *ib.*, pp. 90–96. IV., Elektrometrische Messungen ueber die Bindung des Silbers und des Kupffers an Saccharase und an andere organische Verbindungen, *ib.*, pp. 142–183.

McGuigan, Hugh

'04 The Relation between the Decomposition Tension of Salts and their Antifermentation Properties. The American Journal of Physiology, Vol. X., pp. 444–451.

Lillie, Frank R.

'14 Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*. Journal of Exp. Zoöl., Vol. 16, pp. 523–590.

'19 Problems of Fertilization. xii + 278 pp. The University of Chicago Press.

'21 Studies of Fertilization. VIII. On the Measure of Specificity in Fertilization between two Associated Species of the Sea-Urchin Genus Strongylocentrotus. BIOL. BULL., Vol. 40, pp. 1–22.

Just, E. E.

'19 The Fertilization Reaction in *Echinarachnius parma*. I. Cortical Response of the Egg to Insemination. BIOL. BULL., Vol. 36, p. 1.

PHAGOCYTOSIS AND CLOTTING IN THE PERIVISCERAL FLUID OF ARBACIA.

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The perivisceral fluid of the Echinoidea contains certain formed cellular elements which have been observed and described by Valentin ('42), Williams ('56), Hoffman ('72), Prouho ('86), Geddes ('80), Cuénot ('91) and Théel ('96). For most of our knowledge of these cellular elements we are indebted to Geddes and Cuénot. Goodrich ('19) in his discussion of the leucocytes of the invertebrates is the most recent contributor.

Cuénot observed that these formed elements were found not only in the perivisceral cavity, but also in the lumina of the ambulacral system; in the lacunæ of the intestinal walls and in all other organs of the body. He further observed that they were not all of one type, but could be divided into two distinct series, each of which series could be further subdivided. The first series of cells he called "vibratile globules," minute cells 6–9 micra in diameter having a head and vibratile tail. The head is mostly nucleus enclosed in a thin sheath of cytoplasm. According to Cuénot the function of this type of cell is to keep the fluid contents of the perivisceral cavity in motion.

The second series of cells were called "amibocytes" and were subdivided into several different types. The first type, from which Cuénot assumed the other types to be derived, was found abundantly in the lymph-glands. The cells of this type were characterized by a homogeneous cytoplasm, long and numerous pseudopodia, and a relatively large nucleus within which was a distinct nucleolus. Sometimes the amibocytes of this type enclosed yellow or brown refractile granules. According to Geddes these were the most numerous of the corpuscles and all of the investigators have agreed that these cells are the chief agents in the formation of the blood clot. Because of this activity

Dekhuyzen ('01) named them "thrombocytes" and compared them to the blood platelets of the vertebrates. Cuénot, Geddes and Dekhuyzen believed that when a clot was formed, these cells fused with each other to form larger true plasmodial masses, the cells of such a mass losing their identity. In order to check this assumption, Geddes introduced colored granules into the perivisceral fluid and observed that these colored granules were to be found in the large multinucleate masses which were formed by the fusion of the amibocytes. Michel ('88) and Goodrich ('19) claim that these cells do not form true plasmodial masses, but do form aggregate plasmodia in which the cells retain their identity and are in contact by their ectoplasmic surfaces alone. Goodrich states further that these cells, instead of having a number of delicate pseudopodia, are under normal conditions provided with numerous flap-like membranous processes similar to the undulating membranes of the Protozoa. These membranous flaps when viewed in optical section have the appearance of delicate pseudopodia. The membranous flaps are constantly changing and when a drop of the perivisceral fluid is taken out of the cavity and spread out on a slide, these cells clump together if approximate to each other; if isolated, the membranes shrink as the fluid evaporates leaving long, filamentous strands of ectoplasm.

The other types of amibocytes are larger than those just described and contain colored or colorless globules. They also have a relatively smaller nucleus and form blunt pseudopodia when moving in the perivisceral fluid. Various functions have been ascribed to these larger amibocytes. The red, green and yellow material appearing in these amibocytes was said by Cuénot to be stored fat; by McMunn to be a respiratory pigment which he called by the non-committal name of echinochrome; and by Ribaucourt this colored material was thought to be excretory matter which was to be carried out of the body through the ambulacral system by these amibocytes. They were all agreed that these amibocytes were derived from the first type of amibocyte by the inclusion of this material or by its precipitation after absorption. Since these nutritional or nephritic

cells take but a passive part in the formation of a clot and none at all in phagocytosis, they will not be considered further. The name leucocyte which Goodrich applied to the first type of amibocyte will be used here to designate the type of amibocyte active in the process of phagocytosis and clotting.

The leucocytes of *Arbacia* have the same general appearance as those figured by Goodrich for *Asterias*, but the membranes of the leucocytes of *Arbacia* seem to be larger in comparison to the amount of endoplasm than do those of *Asterias*. As the leucocyte moves about in the plasma medium the membranes are constantly changing (Fig. 1, *a*, *b*, *c*). In the living condition,

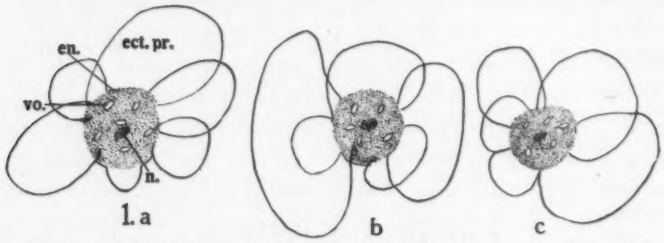

FIG. 1, *a*, *b*, *c*. Leucocyte of *Arbacia* moving freely in plasma medium, drawn at five-minute intervals. Semi-diagrammatic. × 1,500. *ect. pr.*, ectoplasmic process; *en.*, endoplasm; *n.*, nucleus; *vo.*, vacuoles.

the cytoplasm of the leucocyte appears finely granulate and contains five or six hyaline vacuoles. The nucleus is scarcely discernible in the living cell, but when treated with Flemming's or Herman's fluid it appears as a large ovoid body with a very distinct membrane. The chromatin granules are very prominent in the nucleus of those cells stained with Haidenhain's

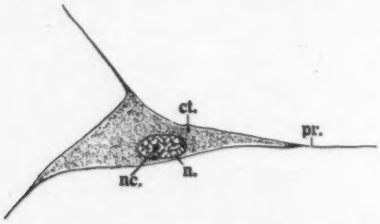

FIG. 2. Leucocyte of *Arbacia* from clot. Semi-diagrammatic. × 2,500. *ct.*, cytoplasm; *n.*, nucleus; *nc.*, nucleolus; *pr.*, ectoplasmic process.

hæmatoxylin. A large nucleolus is also present. When fixed with Schaudinn's fluid and stained with Haidenhain's hæmatoxylin, the cytoplasm appears reticulate (Fig. 2).

I. Phagocytosis.

Carmine and india ink suspensions in sea-water were used in order to determine the extent to which the leucocytes took part in the inclusion of foreign particles from the perivisceral fluid. One cubic centimeter of sea-water suspension of finely powdered carmine was introduced into the perivisceral fluid through a perforation in the peristomial membrane. The individual so treated was put back into the aquarium and at the end of thirty minutes a drop of the perivisceral fluid was examined. Although a large number of carmine grains were yet free in the plasma, it was observed that all of the leucocytes had taken up one or more particles. At the end of an hour another examination was made of the perivisceral fluid and at this time the plasma was comparatively free from carmine particles and the leucocytes were heavily loaded with them. The ingestion of several of these particles by the leucocyte was observed. This individual was kept in the aquarium for two weeks during which time the perivisceral fluid was examined at intervals. At the end of two weeks some leucocytes containing carmine particles were still present in the perivisceral fluid. This fact indicates a period of longevity on the part of these cells, but the fate of the large number which performed the function of phagocytes is still in doubt. Ribaucourt suggested that after they had become loaded with waste material from the perivisceral fluid they made their way into the ambulacral system by diapedesis and thence passed out through the madrepore. Since, however, no currents have been observed nor any cells observed leaving the madrepore, this assumption is unconvincing. The problem of excretion and with it the fate of the old phagocytes has yet to be solved.

The results obtained by the injection of 1 c.c. of india ink-sea-water suspension were the same as those obtained with the carmine suspension. And further in the study of the clot, it was observed that the ingestion of particles by the leucocytes does not inhibit their activity in the formation of the clot.

In both of these experiments the amibocytes other than phagocytes did not act as phagocytes. This may be due to the fact that, if they are, as assumed, derived from leucocytes in the first place by the inclusion of reserve food material or absorption of nitrogenous waste, the material so added inhibits any phagocytic activity.

II. Reactions to Vital Stains.

No success was met with in the attempt to stain the leucocytes by the injection of weak intravitam stains. Aqueous solutions of methyl blue, methyl green, Janus green, and trypan blue were introduced into the perivisceral cavity, but no effect on the formed elements was noted. A 1:500 methyl blue-sodium chloride solution had no effect upon the leucocytes, but faintly stained the globules in the red amibocytes ("globules amœboide brun d'acajou" of Erdle).

One c.c. of .01 per cent. neutral red in sea-water was injected into the perivisceral fluid of several individuals and after twenty-four hours all of the leucocytes in several drops of the perivisceral fluid examined contained yellow globules. This indicated that the hyaline globules apparent in the leucocyte under normal conditions have an alkaline content.

III. Clotting.

A drop of the perivisceral fluid of *Arbacia*, freshly drawn, when placed on a cover slip immediately shows signs of agglutination. The large pigmented and colorless amibocytes appear in long strings held together by the clumping of the leucocytes, although in many cases the leucocytes had not lost their membranous processes. A drop of the perivisceral fluid was placed on a glass slide and observed continually for two hours. Sketches of the changes which were taking place in the shape of the cells in a restricted area were made at intervals of thirty minutes in order to show the rôle played by the leucocytes in the formation of the clot. When first observed there were four leucocytes in the field, each possessing the typical membranous projections (Fig. 3). As the fluid evaporated during the course of an hour these leucocytes came into contact with the slide.

Whereupon the membranous processes of the ectoplasm began to shrink and the leucocyte to elongate (Fig. 4). Several red, yellow and colorless amibocytes which were in the vicinity

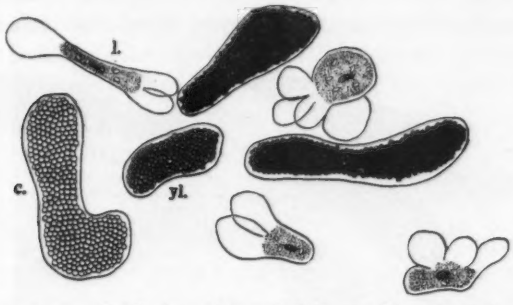

FIG. 3. Group of cells in perivisceral fluid on slide. Semi-diagrammatic. × 1,000.

adhered to the margins of the leucocytes as if the latter were secreting a sticky substance. At the end of one and a half hours the leucocytes had become extremely elongate and the membranous projections had entirely disappeared (Fig. 5). The filamentous projections left from the membranes extended across the field and came into contact with those of the neighboring leucocytes so that a meshwork was established to which the larger pigmented and colorless amibocytes adhered.

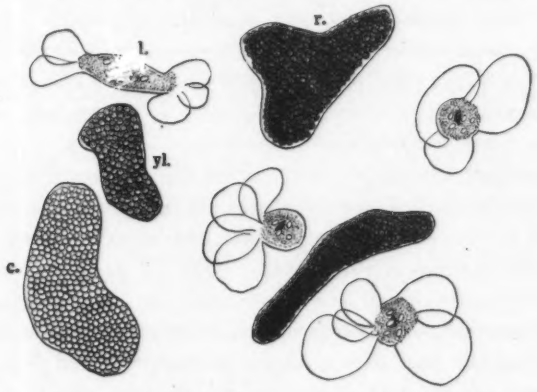

FIG. 4. Same group of cells as in Fig. 3, drawn 30 minutes later to illustrate beginning of clot formation. Semi-diagrammatic. × 1,000.

The activity of the leucocyte was then observed in a hanging drop, but typical clot formation did not occur except where the leucocytes came in contact with the glass. If the leucocytes in this drop were isolated from each other they retained their membranous projections, but if they came into contact with

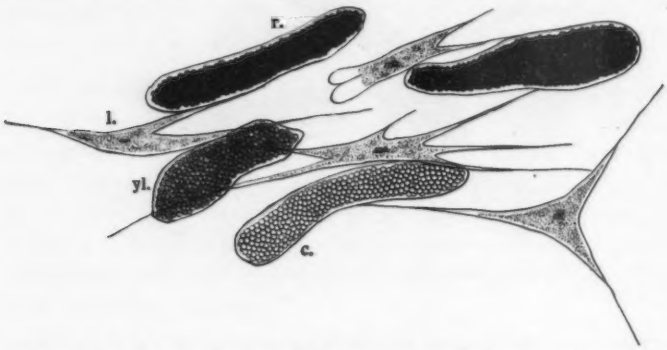

FIG. 5. Same group of cells as in Figs. 3 and 4. Clot fully formed. Drawn 30 minutes later than Fig. 4. Semi-diagrammatic. × 1,000. *c.*, amibocytes with colorless globules; *l.*, leucocyte; *r.*, amibocyte with red globules; *yl.*, amibocyte with yellow globules.

each other they tended to stick together and form a mass to which the other amibocytes adhered. A meshwork such as observed under the conditions of evaporation did not appear. I was unable however to observe whether or not the leucocytes formed true plasmodia or only aggregate plasmodia. If their reaction to each other in forming these masses is similar to the manner in which they form the meshwork of the clot then they must certainly retain their individuality and come into contact without cytoplasmic fusion.

An attempt was made to find out the rôle the leucocyte played in the regeneration of resected parts. In the case of a perforation or a slit in the peristomial membrane, the leucocytes form a clot along the margins of the wound. This clot gradually extends across the opening and eventually closes it. If a piece of the test is removed, the leucocytes form a clot around the margin of the perforation and after a longer or shorter period depending upon the size of the piece cut out, this clot closes it. The

further history of the relation of the clot to the tissues originally occupying the resected area will be followed later.

SUMMARY.

1. Phagocytosis in the perivisceral fluid of *Arbacia* is carried on by the leucocytes alone. These cells possess membranous processes similar to those of the leucocytes of *Asterias* and are the smallest and most numerous of the amibocytes. As phagocytes the leucocytes have a long period of activity.

2. The leucocytes tend to agglutinate even in freshly drawn perivisceral fluid. In a drop of perivisceral fluid on a plane surface, the leucocytes lose their characteristic membranous processes upon contact with the surface and become elongate. Long filamentous processes are produced by the shrinkage of the membranous processes. These filamentous processes adhere to those of neighboring leucocytes forming a delicate mesh to which the pigmented and colorless amibocytes adhere.

3. The leucocytes show an alkaline reaction when treated with neutral red in sea-water, but are not affected by weak solutions of intravitam stains introduced into the perivisceral cavity.

4. If a piece of the peristomial membrane or the test is resected, the leucocytes form a clot which closes the wound.

BIBLIOGRAPHY.

Cuénot, L.

'89 Etudes sur le sang, son role et sa formation dans la serie animale. Partie 2. Invertebrés. Note preliminaire. Arch. Zool. exper., Ser. 2, Tome 7: 1–9.

'91 Etudes Morphologiques sur les Echinodermes. 8 Taf. Arch, Biol., Tome 11: 303–680.

'91 Etudes sur le sang et les glandes lymphatiques dans la serie animale. Partie 2. Invertebrés. Arch. Zool., Ser. 2, Tome 9: 613–641.

Dekhuyzen, M. C.

'01 Ueber die Trombocyten (Blutplättchen). Anat. Anz., 19: 529–540.

Geddes, P.

'80 Observations sur le fluide perivisceral des oursins. Arch. de Zool. exp. et gener., Tome 8: 483–496.

Goodrich, E. S.

'19 Pseudopodia of the Leucocytes of Invertebrates. Quart. Jour. Micr. Sci., Vol. 64: 19–27.

Griffiths, A. B.

'92 Sur l'echinochrome: un pigment respiratoire. Compt. Rend. Acad. Sc. Paris, Tome 115: 419–420.

Kollmann, M.

'08 Recherches sur les Leucocytes. Ann. Sc. Nat. Zool., Ser. 9, Tome 8.

List, Th.

'97 Ueber die Entwickelungen von Proteinkrystalloiden in den Kernen der Wanderzellen bei Echiniden. Anat. Anz., 14: 185–191.

Michel, A.

'88 Sur la pretendue fusion des cellules lymphatiques en plasmodes. Compt. Rend. Acad. Sci. Paris, Tome 106.

McMunn, C. A.

'85 On the Chromatology of the Blood of Some Invertebrates. Quart. Jour. Micr. Sci., Vol. 25: 469–490.

Prouho, H.

'86 Sur le Systeme vasculaire des Echinides. Compt. Rend. Acad. Sci. Paris, Tome 103: 560–563.

Schäfer, E. A.

'83 On the Perivisceral Fluid of the Sea Urchin (*Echinus*). Proc. Roy. Soc. London, Vol. 34: 370–371.

Théel, H.

'96 Remarks on the Activity of Amœboid Cells in the Echinoderms. Festsch. Lilljeborg Upsala. pp. 47–58.

Valentin, G.

'42 L'Anatomie du genre *Echinus*. In L. Agassiz's Description des Echinodermes vivans et fossiles.

Williams, Thos.

'56 On the Mechanism of Respiration in the Family of Echinidæ. Rep. Brit. Assoc. Adv. Sci., 26th Meeting.

STUDIES ON THE PARASITIC WASP, HADROBRACON BREVICORNIS (WESMAEL).

II. A Lethal Factor Linked with Orange.

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Linkage of genetic factors has been demonstrated in many widely diverse species of plants and animals. In animals it may be complete in either sex, partial in the other; or it may be partial in both sexes. In plants it is apparently partial in both types of sporogenesis. Up to the present time linkage has not been demonstrated in Hymenoptera. In species which produce males by haploid parthenogenesis we might expect linkage in ovogenesis to be partial. In spermatogenesis linked factors would, of course, be completely linked as in the case of sex-linked factors in *Drosophila*. Unlike sex-linkage, however, there would be several independently segregating sex-linkoid groups, corresponding to the reduced number of chromosomes.

In the parasitic wasp, *Hadrobracon*, orange eye color is inherited as a sex-linkoid recessive to typical black. Heterozygous females produce black and orange males in equal numbers as previously shown.[1]

Three heterozygous sisters were isolated in July, 1920. One, a virgin, produced 38 black males and 44 orange males.

The second was bred as a virgin and later mated to one of her orange-eyed impaternate sons so that females were produced. Her progeny consisted of 57 black males, 44 orange males, 37 black females and 29 orange females. One of her black daughters isolated as a virgin produced 14 black males and 16 orange males.

Under normal conditions full-grown larvæ of the wasp spin cocoons before pupating which serve to attach them firmly in

[1] Whiting, P. W., "Studies on the parasitic wasp, *Hadrobracon brevicornis* (Wesmael)—I., Genetics of an Orange-eyed Mutation and the Production of Mosaic Males from Fertilized Eggs," Biological Bulletin, Vol. XLI., No. 1.

the culture vial. If the supply of food is insufficient some of the larvæ pupate without spinning cocoons and are shaken into the ether bottle when the first count is made. If returned to the vial they will, in most cases, mature at the expected time.

The progenies of the heterozygous females above recorded consisted of black males, 104 adults, 5 pupæ; orange males, 101 adults, 3 pupæ; black females, 37 adults, no pupæ; orange females, 28 adults, 1 pupa. The ratio is quite in agreement with expectation and nothing unusual was noticed about the pupæ. Some of them would probably have metamorphosed to adults had they been replaced in the culture vial.

The third heterozygous black sister isolated as a virgin-produced offspring in an unexpected ratio. It was first noticed that the adult progeny were almost all orange and that there was an unusual number of small-sized pupæ without cocoons. Examination of the latter, moreover, showed that most of them had black eyes. It was immediately suspected that there was a lethal factor coupled with the normal allelomorph to orange. Consequently all pupæ were kept beyond the normal time of eclosion and those that failed to mature were counted as lethal. The black-eyed mother was mated to an orange son in an attempt to get black-eyed daughters carrying the lethal. The total progeny of this lethal-bearing female consisted of black males, 4 adults, 28 pupæ; orange males, 44 adults, 4 pupæ; black females, 2 adults, 3 pupæ; orange females, 18 adults, no pupæ. Among the females the excess of orange over black adults and the presence of black pupæ might seem to indicate partial dominance of the lethal factor. In any case the males show a striking departure from expectation. Black pupæ and orange adults would be *straights* and black adults and orange pupæ would be *crossovers*, except that a few of the pupæ might have failed to eclose on account of partial starvation. Among the males of this one fraternity there were 8 crossovers and 72 straights or 10 per cent. crossovers.

The two black-eyed daughters were isolated and later bred to orange sons. One produced black males, 5 adults, 17 pupæ; orange males, 23 adults, 6 pupæ; black females, 2 adults, 2 pupæ; orange females, 2 adults, no pupæ.

The other produced black males, 11 adults, 27 pupæ; orange males, 30 adults, 11 pupæ; black females, 3 adults, 1 pupa; orange females, 3 adults, 2 pupæ.

The lethal factor is therefore inherited, there being in this generation 33 crossovers and 97 straights or 25 + per cent. crossovers.

Although considerable care was taken to preserve the lethal stock it very rapidly died out due to difficulty in getting black-eyed lethal-bearing females, and moreover the total numbers in the fraternities became very small. Only three black-eyed females had offspring in the next generation and these were all males. One produced black, 5 adults, 27 pupæ; orange, 20 adults, 6 pupæ.

A second produced black, 3 adults, 12 pupae; orange, 15 adults, 4 pupæ.

The third produced all adults, 5 black and 9 orange. Although the numbers are very small in this last-mentioned fraternity the failure of any lethal pupæ to appear in spite of the presence of 5 adult black males is good evidence that the mother originated from a crossover or non-lethal black-bearing egg.

The total male progeny of all five lethal-bearing females is black, 28 adults, 111 pupæ; orange, 132 adults, 31 pupæ; 59 crossovers to 243 straights, or 19.5 per cent. crossovers.

. Partial linkage is therefore demonstrated in Hymenoptera.

During the experiments above recorded a number of the lethal pupæ were fixed in Carnoy's fluid for histological examination. Sagittal sections, stained with iron hæmatoxylin, showed no abnormal growths or defects in any organs. The nature of the lethal effect is therefore unknown.

EXPERIMENTS ON THE DEVELOPMENT OF THE GILLS IN THE AMPHIBIAN EMBRYO.[1]

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The present work upon the gills was undertaken as a continuation of the study of relations of symmetry in embryonic organs, in the hope that it might throw light on questions raised in previous studies of the limbs.[2] The external gills of the salamander were chosen to this end, as constituting another system with right and left enantiomorphs, each without any plane, axis, or center of symmetry in itself. Owing to the complexity of the system, however, if for no other reason, the experiments failed to give as clear evidence on the questions raised as the limbs had done. Nevertheless, they have brought to light certain interesting facts, which in part corroborate and in part extend those reported by Ekman ('13), who worked upon various anuran embryos. They show, furthermore, certain differences between *Amblystoma* and the anurans with respect to the organs studied.

The gill arches and external gills form a complex in which all of the germ layers are represented. There is an outer covering of ectoderm, a central core of mesoderm, which contains the blood vessels and extends out into the gill filaments, and an inner lining to the arches and the clefts consisting of endoderm. Besides these the ganglion crest contributes elements to the arches, chiefly to the formation of the cartilaginous skeleton.

The experimental work of Ekman upon anurans has shown the following: (1) That when the ectoderm covering the gill region is removed, the regenerated ectoderm is capable of forming gill filaments. (2) That when the gill ectoderm is replaced by ectoderm from another region, with certain exceptions, the for-

[1] Read before the National Academy of Sciences, November 17, 1920

[2] Harrison ('17 and '21).

mation of gills is suppressed. (3) That the external gills and the outer branchial grooves are specifically determined in the ectoderm and that the endoderm plays no part in the localization of the gills. (4) That the ectodermal rudiments of the gills have, from early stages on, a certain power of self-differentiation which varies amongst the several species studied. (5) That gills which arise from displaced ectoderm, together with atypical endoderm, conform entirely as to their position, form, and size, to the ectoderm. (6) That the branchial blood vessels are an essential factor in the later development of the gills.

The present experiments were made upon the embryo of a urodele, *Amblystoma punctatum*. They concern almost entirely the two outer layers, ectoderm and mesoderm. Owing to technical difficulties, the endoderm was experimented with only to a very limited extent, and this layer, therefore, does not figure in the present analysis. While certain things are quite definite, the work must be considered as incomplete, inasmuch as there has been no opportunity as yet to examine the specimens in sections.

With regard to the technique of operation, only one thing requires special mention. The donor embryo was in most cases stained *intra vitam* in Nile blue sulphate, which colors the ectoderm bright blue.[3] The stain persists for many days, so that the exact delimitation of the graft can be observed, as development proceeds. This removes a serious source of error, for it is thus always possible to determine whether the parts under observation are derived from the host or the graft. Embryos from stage 21 (Fig. 1, medullary folds just closed) to stage 25 (just before appearance of tail bud[4]) were used for nearly all the experiments. A few were made with earlier and a few with slightly later stages. Subsequent experiments made with still later stages show that marked changes in the behavior of the ectoderm after transplantation occur at about the time when the mesectoderm from the ganglion crest grows down and the first branchial pouch is developed (stage 29). These will not be considered at present.

[3] Detwiler ('17).

[4] Stages 25 and 29 have been figured previously (Harrison '18, Figs. 1 and 2).

Removal of the Ectoderm of the Branchial Region and its Replacement by Ectoderm from Another Part of the Body.

The gill region in an embryo with closed medullary folds is shown in outline in Fig. 1 (*BR*). If this ectoderm is removed and replaced by ectoderm from the flank (*FL*) or any other part except that immediately surrounding the gill region, complete suppression of the operculum and the gills on the operated side (Fig. 2) results. Thus, out of twenty-four cases in which ectoderm from the flank was used, seventeen had the gills entirely lacking. In six cases in which the third gill developed and in one in which the first gill was formed, ectoderm from the host covered the greater part of the gill including the tip.

When the covering ectoderm is taken from the region just behind the gills, *i.e.*, from the pronephric and limb area (*PN*), functional gills develop. This was found in five cases, in three of which all three gills were well developed, while in the two others the first gill was either absent or rudimentary.

When, however, the ectoderm is taken from the ventral mid-region (*CD*), *i.e.*, from the region immediately ventral to the branchial, the result is not so favorable. Out of nine cases, no gills at all developed in four, a functional third gill in one, a functional third and rudimentary second in three, and two functional gills (second and third) in one.

Likewise, when the covering is taken from the anterior part of the head, there is usually a partial development of the gills, involving principally the second and third. The transplanted piece was taken in various ways (Fig. 1, *C*, *C'*, *A*). In general, it may be said that the closer the seat of origin of the graft is to the normal branchial region, the more perfect are the gills that develop. A supernumerary balancer developed in almost all of the cases in which the graft included the normal balancer ectoderm.

When the ectoderm covering the gill region is removed and the wound left uncovered, as it was in the donor embryos in many experiments, the surrounding ectoderm pushes over the wound, covering it usually in twenty-four hours or a little more.

The mortality among these embryos is high, but when they live, gills nearly always develop from this regenerated ectoderm. In some cases the gills formed are normal; in others, there is a reduction in number or in size or both, the first gill being more often affected than the others.

Bilateral Extirpation of Branchial Ectoderm.

As far as the question of the morphology of the gills is concerned, this experiment is in no wise different from the unilateral operations, but physiologically it may prove of greater interest. At present only a few cases are available for study. In three experiments the branchial ectoderm was removed on both sides and replaced by ectoderm from the flank. In two of these cases no gills at all developed, while in one the first gill on the left side, which was partly covered by ectoderm from the host, was rudimentary. In the last-named case the circulation of the blood was never established and the embryo became œdematous. In both of the other cases the circulation was established in the vessels of the yolk, but the heart action became more and more feeble, and after a time the circulation of the blood ceased entirely. Both of these eventually became œdematous also, though not extremely so. One individual, killed fourteen days after the operation, showed marked lack of sensitiveness to tactile stimuli, and responded to a needle prick only with a single jerk. It was apparently unable to swim. The other could execute swimming movements fairly well, though in a somewhat jerky fashion. A fourth case that should be considered in this connection had the branchial ectoderm removed on both sides after which it was allowed to regenerate without replacement from elsewhere. The operculum was formed on both sides but the gills failed to develop except in most rudimentary form—on the right side two rudimentary gills and on the left only a sharp conical process (Fig. 3). Blood circulating in these gills was first observed thirteen days after the operation. The larva was kept under observation for thirty-six days, at which age it was preserved. Its development, apart from the absence of gills, was normal.

The experiments are too few in number to warrant very definite conclusions. Absence of gills from both sides seems, in some cases at least, to lead to weakened heart action and ultimate stoppage of the circulation. On the other hand, the last case shows that it is possible for the embryo to adjust itself to the almost complete loss of its respiratory organs, sufficient aeration of the blood probably taking place through the general surface of the body.

Change of Orientation of the Branchial Ectoderm.

These experiments were for the purpose of determining whether there is a definite polarization of the ectodermal elements, and, further, whether any specific gill pattern is predetermined in the region. The experiments of the preceding sections already indicate that the latter is not the case.

Transplantations were made in four ways, as was done in the case of the experiments with limbs. The ectoderm was removed from the gill region and replaced by similar ectoderm either from the same or from the opposite side of the body and placed either with the dorsal or with the ventral border of the graft corresponding to the dorsal border of the wound. Besides these a few experiments were made in which the graft was rotated only 90°. The results may be stated very simply, for there is a marked tendency toward normal development in all combinations. Forty-eight experiments were made, twenty-eight of which gave definite results. Thirteen yielded normal gill-complexes, and these were distributed amongst all of the six different orientations tried. In five cases the second and third gills were normal but the first was small; in two cases the first gill was rudimentary while the others were normal; and in three cases the first gill was altogether lacking, the other two being normal. There were only three cases in which the gill-complex was irregular.

From these experiments the conclusion may be drawn that the branchial ectoderm is equipotential, and that there is no polarization of the elements affecting the potencies of differentiation. In this respect the branchial ectoderm of *Amblystoma* is markedly different from that of the anurans studied by Ekman.

In the latter changes in orientation of this layer are followed by corresponding changes in the position of the gills. This lack of axial differentiation in the gill ectoderm of the *Amblystoma* embryo lasts, however, for a brief period only, for in slightly later stages (from stage 29 on) change in orientation of the ectoderm is followed by marked disturbances in the arrangement of the gills.

Removal of the Mesoderm of the Branchial Region.

No satisfactory method of removing the mesoderm of the gill region and replacing it by other mesoderm has been worked out. However, it is a simple matter to remove all of this layer from the region in question and to cover the wound with clean ectoderm from the branchial region of another embryo. This affords the surrounding mesoderm an opportunity to wander in and fill the place taken by the original branchial mesoderm. The wound after such operations heals, though not nearly so readily as when there is mesoderm underlying the outer layer.

Sixteen such experiments were made, of which ten were successful. In only two of these cases was there any radical disturbance of the normal development of the gills. The other eight individuals developed approximately normally, though in five of them the heart was affected and the circulation was never established in the embryo. In three cases, however, the circulation was established on both sides, the gills of the operated side remaining smaller than the normal ones. In one the first gill was suppressed.

From the fact that in such a large proportion of cases the development of the circulation was radically interfered with, it seems likely that the removal of the mesoderm must have disturbed the material that goes to make up the heart. Aside from this the effect of the operation is slight. The mesoderm of the surrounding region obviously has the potency to fill in the gap made by the removal of tissue and then to take part in the development of normally formed, though at first smaller, gills.

Change of Orientation of the Branchial Ectoderm and Mesoderm.

These experiments are similar to those in which the ectoderm alone was turned, differing only in the circumstance that both ectoderm and mesoderm were lifted and implanted together. There is no difficulty in separating the mesoderm from the endoderm, though the healing of the wounds after transplantation is much less regular than when the ectoderm alone is taken. Inasmuch as turning the ectoderm alone has no effect upon the development of the gills, the effect of the transplantation of the two layers together must be due to the mesoderm alone. The respective results are strikingly different after the two operations.

There were sixty-three experiments in which both mesoderm and ectoderm were transplanted. Thirty-seven were positive. These cases were distributed unevenly amongst the different orientations, but the results throughout, except when the graft was from the same side of the body and normally oriented, were extremely variable and difficult to place in distinct categories.

The normally oriented grafts (homopleural dorsodorsal) gave five normal or nearly normal cases and one distinctly abnormal individual in which two gills were irregular and fused together.

In none of the other orientations were there any absolutely normal cases. However, in the inverted grafts from the opposite side of the body (heteropleural dorsoventral) there were three approximately normal individuals out of eleven positive experiments. Of the remaining eight cases, no two have been given the same designation except two which have been classified as very irregular and two others put down as rudimentary. Three gills were distinguishable in five of the eight cases, and only one gill was present in one. In one of the eight cases, and possibly in others, the abnormalities consisted in defects which might have been due to general conditions rather than to any particular disturbance of orientation.

Inverted grafts from the same side of the body (homopleural dorsoventral) gave not a single case, in a total of thirteen, that even approached the normal condition. Two gills were de-

veloped in five cases, but they were abnormal in some respects (Fig. 4); in the others only one gill was formed. Two individuals had quite irregular gills and three had rudimentary ones.

In the non-inverted grafts from the opposite side of the body (heteropleural dorsodorsal), of which there were only six available cases, the results were in no two cases exactly alike. In one a nearly normal complex developed. In two others three gills developed, but with abnormalities. The others were still less perfect.

The above results, standing alone, do not lead to any definite conclusion regarding the exact rôle of the mesoderm in the formation of the gills or regarding the nature of the disturbance caused by abnormal orientation of this layer. It is not clear that it is due simply to the reversal of a single polarized axis, such as is the case in the mesoderm of the anterior limb, although the fact that there is a certain tendency toward normal development in the inverted grafts from the opposite side of the body might be taken to indicate that the anteroposterior axis of the elements of this layer is more markedly polarized than is the dorsoventral. The experiments described in the next section point somewhat more clearly to such a conclusion.

Effect of Increasing the Amount of Mesoderm in the Branchial Region. Superposed Grafts.

In this group of experiments the branchial ectoderm was removed without materially injuring the underlying mesoderm, and a piece of tissue of the same size and shape including both mesoderm and ectoderm was healed over the wound. In this way the amount of mesoderm was approximately doubled, there having been only a very slight loss of mesoderm cells through the operation. The grafts were oriented in the four different ways as in the previous experiments.

The group with normal orientation (homopleural dorsodorsal) developed gills that were normal or nearly so in seven cases (Fig. 5), and in only one, in which the three gills were closely fused, was there any marked abnormality.

The inverted grafts from the opposite side of the body (hetero-

pleural dorsoventral) formed in all five cases gills which approached the normal condition. In two of these the gills were distinctly smaller than normal and in the other three there was considerable fusion and some irregularity, though all three gills could be distinguished.

On the other hand, the inverted grafts from the same side of the body (homopleural dorsoventral) showed in all five cases a very defective development of the gills, only one small, irregular or abortive gill being formed in each case.

In the non-inverted grafts from the opposite side of the body (heteropleural dorsodorsal) the results were likewise for the most part irregular or defective. Two fused gills developed in two cases, and in the other three only a single small, irregular or abortive gill was formed.

The results of these experiments show that while the doubling of the mesodermal material has in itself little or no morphogenetic effect, the orientation of the superadded material is of considerable consequence in determining whether aberrations in the development of the gills occur or not. Aside from experiments in which the transplanted material is normally oriented, these disturbances are apparently least marked in the case of inverted grafts from the opposite side of the body, and in this respect there is an approach to the condition found in the mesoderm of the fore limb bud. However, the results of this orientation of the grafted gill mesoderm are not normal with sufficient constancy to term the combination "harmonic", as was done in the case of the limb.

Removal of both Ectoderm and Mesoderm Without Replacement of Either.

This experiment is a by-product of the experiments described in the last two sections. The individuals are the donor embryos of those operations.

With such a large deep wound as is necessary in removing the gill mesoderm, a high mortality was to have been expected. Out of one hundred and six operations, only ten cases survived ten days or longer. Five others were preserved at intervals from an age of two to that of eight days.

The regeneration that takes place after this operation is very imperfect. In four cases a single fairly normal but small gill was regenerated, identified as the first gill in two cases, the second in one and the third in one. In three of these there were, in addition, one or two rudimentary gills. In the other cases either rudimentary gills alone were formed, or else no regeneration of gills took place at all. Blood circulation was established on the operated side in only two individuals. The circulation was defective throughout the embryo in four cases, as shown by the œdema that developed.

The imperfect restitution that takes place in these experiments must be ascribed to the severity of the operation rather than to the removal of anything specifically essential, for when either the mesoderm or the ectoderm alone is removed there is often complete restoration of function and structure.

Transplantation of Branchial Ectoderm.

The branchial ectoderm was transplanted either to the flank or to the anterior part of the head in a few cases, in order to test the potency of this tissue in strange surroundings. In all four individuals of the first group the results were essentially the same. Small nodules, which might possibly be interpreted as incipient gill sprouts, developed, but after a few days they became obliterated. In one case of the second group, where the gill ectoderm was transplanted to cover the eye and mandibular region, no supernumerary gills were formed and even the normal first gill failed to develop. On the other hand in two cases in which the graft was implanted not quite so far forward (Fig. 1, *A*) there were traces of rudimentary gills in the region of the hyoid and mandibular arches respectively. One of these (Fig. 6) showed two supernumerary filaments quite distinctly, in which, however, the circulation failed to become established. In two other cases, operated in a similar way, no supernumerary filaments were observed.

The potency of the gill ectoderm to form gills in any of these abnormal positions is obviously far less marked in *Amblystoma* than in the anuran species studied by Ekman, especially in *Rana fusca*.

TRANSPLANTATION OF BRANCHIAL ECTODERM AND MESODERM.

The results of this experiment are scarcely more definite than when the branchial ectoderm alone is transplanted. There is an initiation of development but it soon becomes arrested.

In five cases the whole of the branchial mound, including both ectoderm and mesoderm but no endoderm, was placed directly behind the normal gills, *i.e.*, in the region of or just ventral to the pronephros, the ectoderm and mesoderm having been previously removed. About the fourth or fifth day a marked prominence was present in the region of the graft, and, in two of the cases at least, distinct gill sprouts showed. There was, however, no further development; the grafted tissue gradually flattened out and became reduced to a slight hump or nodule.

In three other cases the graft was placed in front of the normal gills, in place of the material normally constituting the mandibular and hyoid arches. The results were not very different, except that the original gills of the embryo were to some extent disturbed in their development. No gills ever developed in front of the normal ones and in all three cases the balancer was suppressed.

EFFECT OF LACK OF FUNCTION ON THE DEVELOPMENT OF GILLS.

It often occurs in experiments with amphibian embryos that the circulatory system does not function properly. Sometimes no movement of the blood can be observed even though the heart does beat. The effect of this upon the gills is noticeable; they never expand fully, and, while preserving their essential character, they have an atrophic appearance.

In experiments on the gills in particular it not infrequently happens that, although the embryo is otherwise normal, the circulation in the operated gills is either delayed or fails altogether to become established. The same atrophic appearance is found in such cases.

When the heart is removed a similar condition ensues as regards the gills, but the effects on the embryo as a whole are much more pronounced. It becomes badly swollen and ulti-

mately dies. Operations of this kind were done upon embryos in stages 33 and 34, *i.e.*, just before the heart begins to beat. It is difficult to remove the heart rudiment completely. There is almost always some regeneration, and pulsation is established, which, however, is usually ineffective and in such cases no blood can be seen in movement in the yolk vessels or through the gills. Nevertheless, the latter grow out, and are normally constituted except for their atrophic appearance. The operculum and the gill plates are also formed. As regards the development of their specific form, the gills, like many other structures, are thus independent of function, though the marks of atrophy in the functionless organs are unmistakable.

Conclusion and Summary.

So far as the present experimental analysis has led, the respective rôles of ectoderm and mesoderm in the formation of the gills are as follows:

The branchial ectoderm is specifically gill forming, but surrounding the gill region, and, more particularly, posterior to it, the ectoderm has the potency to form gills in a diminishing intensity as the distance from the gills increases. This is shown both by transplantation experiments and by regeneration after removal of the branchial ectoderm.

The gill pattern is not, however, laid down in the branchial ectoderm, as is shown by the fact that this same ectoderm, when turned in any way or even when taken from the opposite side, gives rise to normal gills notwithstanding.

The specific pattern must therefore be determined by the deeper layers, but in the absence of experiments upon the endoderm, the exact rôles of mesoderm and endoderm, respectively, cannot be determined. The indications are, however, that the endoderm does not play merely a passive part, as Ekman maintains to be the case in the anurans.

The mesoderm cannot be a well-defined mosaic, for doubling of the material by superposition, provided the orientation of the grafted tissue is normal, does not disturb the normal development of the gills. Abnormal orientation of the mesoderm, however,

both after extirpation of the normal mesoderm and in cases of superposition, results in marked aberration from the normal development, showing that this layer is not without axial differentiation. These aberrations from the normal are not specific for any particular orientation, but there is a greater degree of disturbance after the orientations which, in the case of the fore limb, have been found to be disharmonic, *i.e.*, inverted mesoderm from the same side of the head and non-inverted from the opposite side. One of the combinations called harmonic in the case of the fore limb (that in which the graft is normally oriented) gives normal gills, but the other, while yielding a considerable number of cases of approximately normal gills, results on the other hand in a good many irregularities. These circumstances may be taken to indicate that the gill-mesoderm elements are distinctly polarized in an anteroposterior direction and perhaps less markedly so dorsoventrally. The fact that the mesoderm from the periphery of the gill region is capable of forming gills, taken together with the fact that doubling the amount of the material does not disturb normal development, points to the conclusion that this mesoderm is equipotent.

REFERENCES.

Detwiler, Samuel Randall

'17 On the Use of Nile Blue Sulphate in Embryonic Tissue Transplantations. Anat. Rec., Vol. 13.

Ekman, Gunnar

'13 Experimentelle Untersuchungen über die Entwicklung der Kiemenregion (Kiemenfäden und Kiemenspalten) einiger anuren Amphibien. Morph. Jahrb., Bd. 47.

Harrison, Ross G.

'17 Transplantation of Limbs. Proc. Nat. Acad. Sci., Vol. 3.

'18 Experiments on the Development of the Fore Limb of Amblystoma, a Self-Differentiating Equipotential System. Jour. Exp. Zoöl., Vol. 25..

'21 On Relations of Symmetry in Transplanted Limbs. Jour. Exp. Zoöl., Vol. 32.

EXPLANATION OF PLATE I.

Figure 1. Profile view of an embryo of *Amblystoma punctatum*, stage 21. The various flaps of ectoderm used in the transplantations are indicated by ovals. A, includes the mandibular, hyoid and anterior part of the branchial region; BR, branchial region; C, ventroanterior part of head region; C′, head region; CD, cardiac region; Fl, flank; PN, pronephric and limb region. × 10.

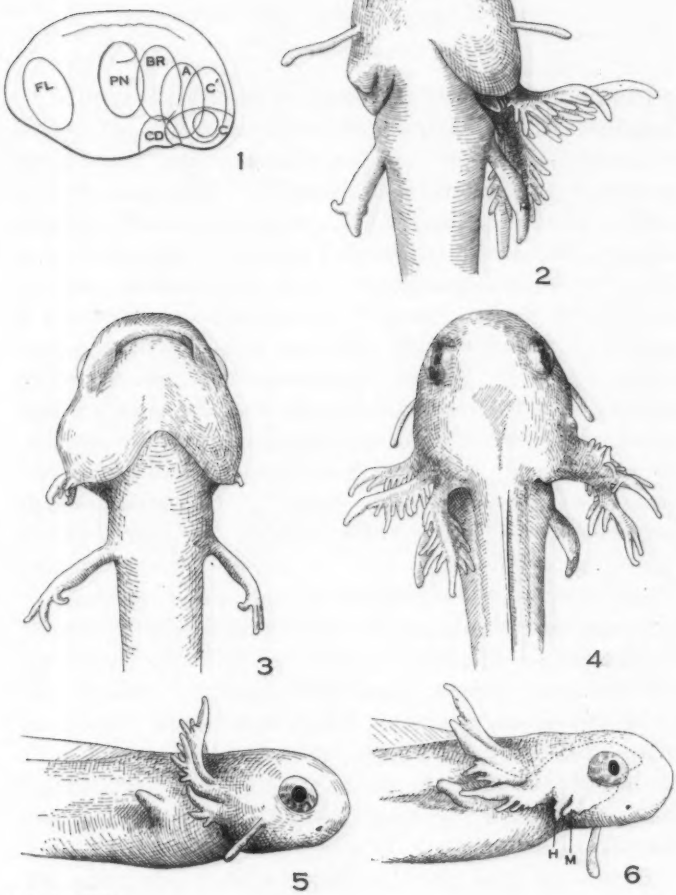
Figure 2. Replacement of branchial ectoderm by ectoderm from the flank (Exp. E. Br. E. 21) resulting in complete suppression of gills on the right side; ventral view of larva eighteen days after operation. × 10.

Figure 3. Larva with gills almost suppressed through failure to regenerate after removal of branchial ectoderm (Exp. E. Br. M 15–); specimen preserved thirty-six days after operation. × 10.

Figure 4. Larva with abnormal gills on the right side due to inversion of the branchial ectoderm and mesoderm (Exp. Br 35), specimen preserved fifteen days after operation. × 10.

Figure 5. Individual showing normal gills developed from double quantity of mesoderm (Exp. S. Br. 17); specimen preserved ten days after operation. × 10.

Figure 6. Individual in which branchial ectoderm (Fig. 1, BR) had been transplanted to a position just anterior to gill region (Fig. 1, A) (Exp. Br. A 2); two supernumerary gills (H and M) developed; the dotted line indicates the boundary of the transplanted ectoderm; sketch made ten days after operation. × 10.



ROSS G. HARRISON.

THE STRUCTURE, LIFE HISTORY, AND INTRA-GENERIC RELATIONSHIPS OF PARAMECIUM CALKINSI, SP. NOV.

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(SEVEN FIGURES.)

The term *Paramecium* was coined by Dr. John Hill of London in 1752 for a group of animalcules comprising "four species," two of which probably include members of the genus *Paramecium* as recognized today.[1] Then O. F. Müller in 1773[2] named an organism *Paramœcium aurelia*, and in 1786,[3] in the first comprehensive attempt to classify scientifically microscopic animals, definitely established the genus *Paramecium* and included under it five species. Unfortunately, however, without the slightest warrant etymological or otherwise, Müller changed the spelling of *Paramecium* to *Paramœcium*. Among the species which Müller distinguished are *Paramœcium aurelia* and *Paramœcium versutum*. The former apparently is Hill's "species 3" and either the present-day *P. aurelia* or *P. caudatum*. *Paramœcium versutum* possibly is Hill's "species 1" and apparently the present species *P. bursaria*. Müller's other paramecia have since been relegated to other genera.

Ehrenberg, in his great monograph on the Infusoria[4] (1838), recognized eight species under the genus and returned to the spelling of Hill with the note "Paramecium ist richtiger als Paramœcium." Among Ehrenberg's species are two true paramecia: *Paramecium aurelia*, which he assumed to be the *P. aurelia* of Müller, and *P. caudatum*, which he distinguished from *P. aurelia* chiefly by the greater attenuation of the posterior end of the animal. Müller's species *P. versutum*, to which Focke in the meantime[5] had given the name *Paramecium bursaria*, Ehrenberg placed under the genus *Loxodes* as *L. bursaria*.

[1] "History of Animals." London, 1752.

[2] "Vermium terrestrium et fluviatilium Historia." Havniae et Lipsiae, 1773.

[3] "Animalcula Infusoria fluviatilia et marina." Havniae, 1786.

[4] "Die Infusionsthierchen als volkommene Organismen." Leipzig, 1838.

[5] "Ueber einige Organisationsverhältnisse bei polygastrischen Infusorien und Rädertieren." *Isis*, 1836.

In 1841 Dujardin[6] recognized only two species of *Paramecium*, *P. aurelia* and *P. caudatum*, and placed *P. versutum* of Müller (= *P. busaria* of Focke = *Loxodes bursaria* of Ehrenberg) in the genus *Bursaria*, though apparently with some hesitancy.

Claparède and Lachmann in their series of studies (1858–1860)[7] distinguished eight species of *Paramecium*, including *P. putrinum*, a new species which is still recognized, *P. bursaria* and *P. aurelia*. They abolished *P. caudatum* with the remark "Le *P. caudatum* Ehr. n'en est certainement pas spécifiquement différent. C'est une simple variété qu'on pourrait même à bon droit considérer comme la forme typique de l'espèce." [8]

Thenceforth, for about thirty years, the name *Paramecium aurelia* was applied indiscriminately to both *P. aurelia* and *P. caudatum*. Most of the pioneer studies on conjugation, etc., were made, as we know now, on *P. caudatum*, though it was called *P. aurelia*. Then came the first hint which was to straighten out the matter. Maupas in 1883[9] noted a difference in the micronuclear apparatus of certain paramecia which led him in 1888[10] to announce definitely that the structure and number of the micronuclei is a distinctive character and thus on this basis to reëstablish the two common species of *Paramecium*. Since 1889, when both Maupas[11] and Hertwig[12] studied the nuclear phenomena during conjugation of these forms, *P. aurelia* and *P. caudatum* generally have been regarded as distinct species, and the validity of this conclusion has been emphasized by Jennings and Hargitt,[13] and by Woodruff[14] using pedigree culture methods.

Studies on the conjugation phenomena of *P. bursaria* by

[6] "Histoire naturelle des Zoophytes, Infusoires, etc." Paris, 1841.

[7] "Études sur les Infusoires et les Rhizopodes," Geneva, 1858–1860.

[8] *Ibidem*, part I., p. 265.

[9] "Contributions a l'etude morphologique et anatomique des Infusoires cilies," *Arch. de Zool. Exp. et Gen.*, (2), I., 1883, p. 660.

[10] "Sur la multiplication des Infusoires cilies," *Arch. de Zool. Exp. et Gen.*, (2), 4, 1888, p. 231.

[11] "Le rajeunissement karyogamique chez les cilies," *Arch. de Zool. Exp. et Gen.*, (2), 7, 1889.

[12] "Ueber die Konjugation der Infusorien," *Abh. Kgl. bayr. Akad. d. Wiss.*, München, 2, Cl. 17, 1889.

[13] "Characteristics of the diverse races of *Paramecium*," *Journ. Morph.*, 21, 1910.

[14] "*Paramecium aurelia* and *Paramecium caudatum*," *Journ. Morph.*, 22, 1911.

Hamburger[15] and of *P. putrinum* by Doflein[16] apparently leave no question in regard to the validity of these species, though both species are remarkably similar.

In addition to *P. aurelia*, *P. caudatum*, *P. bursaria* and *P. putrinum*, which are accepted by Schewiakoff in his monographic survey of the holotrichous Infusoria,[17] there must be mentioned *Paramecium trichium* described by Stokes[18] in 1885, and *P. multimicronucleata* by Powers and Mitchell[19] in 1910.

Stokes discovered *P. trichium* in the zoöglœa on an infusion of dead leaves. His description, in part, is as follows:

"It seems to be a distinct species, and one that can scarcely be mistaken for any known form, except possibly for *P. bursaria* (Ehr.) S. K., differing from the latter, however, conspicuously in form, especially in the apparently oblique curvature of the anterior extremity, in the absence of the truncation of the same part, the absence of the rapid and continuous circulation of the endoplasmic contents, and particularly the green coloration of the cortex and sarcode. The two contractile vesicles, instead of being placed one in each body-half, as in *P. bursaria*, are here anterior and close together, contracting quickly, the one beginning to again form almost before the completion of the other's systole. Trichocysts are very abundant, and are so arranged that they seem to elevate the cuticular surface into the minute hemispherical bosses that cover the entire body. When forcibly extruded through the influence of the glycerole of tannin, the distal end of each, for about one tenth of the entire length, is conspicuously thickened, so that the trichocyst seems to be supplemented by the addition of a minute pyramid."

Schewiakoff considers *P. trichium* to be identical with *P. putrinum*, but if Stokes' description is accurate there is no warrant for this conclusion. Certainly further data are needed before a final answer can be given.

[15] "Die Konjugation von *Paramecium bursaria* Focke," *Arch. f. Protistenk.*, 4, 1904.

[16] "Lehrbuch der Protozoenkunde," 2 Auflage, Jena, 1909.

[17] "Organization et classification des infusoires *Aspirotricha* (*Holotricha auctorum*)," *Mem. Acad. Imp. Sci.*, St. Pétersbourg, Classe d. Sci. Phys. et Math., (8), 4, 1896.

[18] "Some New Infusoria," *Amer. Naturalist*, XIX., 1885.

[19] "A New Species of *Paramecium* (*P. multimicronucleata*) Experimentally Determined," BIOL. BULL., 19, 1910.

P. multimicronucleata, as the name indicates, characteristically has many small micronuclei, usually from six to nine, the structure of which clearly resembles that of the micronuclei of *P. aurelia*. The authors' figures show a general cell-form which is more or less intermediate between that which is typical for *P. aurelia* and for *P. caudatum*. Powers and Mitchell emphasize the persistence of the specific characters after conjugation. It is an interesting form which warrants further study, especially in view of the fact that we now know that reduplication of micronuclei occurs during endomixis.

In January, 1919, I isolated a ciliate, from an infusion of fresh water and vegetable débris collected at New Haven, which, though undoubtedly a *Paramecium*, showed characters that distinguished it from any of the species hitherto described. It has now been extensively studied in pedigree cultures for more than a year and it has bred true. I have, therefore, definitely designated it a new species, and named it *Paramecium calkinsi*[20] in recognition of the fact that Professor G. N. Calkins of Columbia University introduced students of the Infusoria to exact daily isolation pedigree culture methods. *Paramecium calkinsi* presents an interesting composite picture of characters of *P. aurelia*, *P. bursaria*, *P. putrinum* and *P. trichium*, with certain distinctive ones of its own.

The general body form of *Paramecium calkinsi* is more similar to that of *P. bursaria*, *P. putrinum* and *P. trichium* than to that of *P. aurelia* and *P. caudatum*. Indeed, at first glance, one is reminded of *P. bursaria*, and further study suggests *P. putrinum* and *P. trichium* because individuals in different stages of nutrition and dividing at various rates exhibit quite a different appearance. The form which is most characteristic—in fact diagnostic—when the living cells are studied day after day in pedigree cultures is given somewhat diagrammatically in Figs. 1 to 4.[21]

The cells of this pedigree culture of *P. calkinsi*, under favorable food conditions, average about 120 μ in length and 50 μ in

[20] L. L. Woodruff, "*Paramecium calkinsi*, sp. n.," *Proc. Soc. Exp. Biol. & Med.*, Feb., 1921.

[21] Drawings made by Miss J. E. Lovett of this Laboratory.

breadth. In other words, the length is about the same as that of many races of *P. aurelia*, but the breadth is proportionally

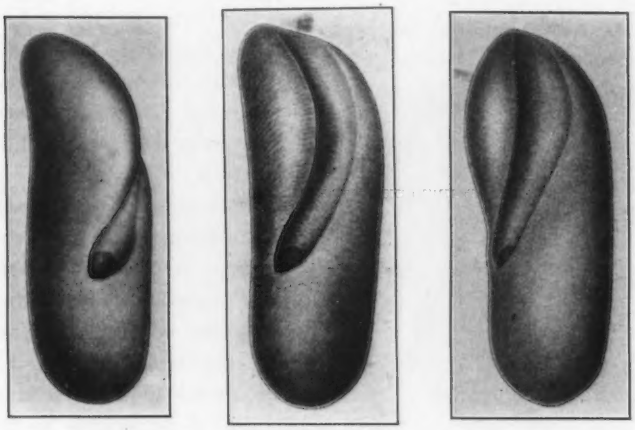

FIG. 1. FIG. 2. FIG. 3.

FIGS. 1–3. *Paramecium calkinsi*, sp. nov.

greater and the broadest part is anterior, whereas it is posterior to the middle in *P. aurelia*. But, of course, no emphasis can be placed on size relations since it is probable that when *P. calkinsi* from other localities is studied, racial size differences will be found to occur.

The ciliary apparatus of the new species affords no striking characteristic and an undulating membrane is present in the gullet as in all paramecia. The trichocyst apparatus is particularly well developed. The trichocysts are relatively evenly distributed in the cortical layer of protoplasm, except at the anterior end where they are more closely crowded together. The surface of the cell in profile presents, upon careful study, a slightly crenulated outline, each of the tiny elevations representing the position of a trichocyst and the depressions between affording the points of origin of the cilia. The structure of the trichocysts as they appear in situ is shown in Fig. 4. Exploded and detached examples appear in Fig. 5.

It will be noted that the trichocyst apparatus of *P. trichium*, as decribed by Stokes, and which he emphasized in naming the organism, agrees closely with that in the new species. The one essential difference is the form of the exploded trichocysts, which in *P. calkinsi* have almost the identical structure of those in *P. caudatum* as described by Khainsky.[22]

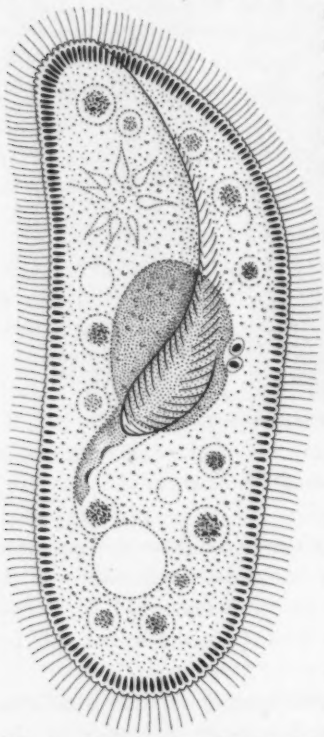

FIG. 4. *Paramecium calkinsi*, sp. nov.

The endoplasm of the new species is characteristically more vacuolated than that of *P. aurelia* or *P. caudatum* when all are bred in the same culture medium. On the other hand it is by no means so fluid as in *P. bursaria*. It is possible to distinguish almost at a glance the new species from the others, merely by the cytoplasmic appearance, when all are mingled together in the same culture.

The contractile vacuoles, as the figure shows, are two in number, and agree closely in position and form with those typical of *P. aurelia*, *P. caudatum*, and *P. bursaria*. Herein is a striking difference from *P. putrinum* with one contractile vacuole at the anterior end and *P. trichium* in which two contractile vacuoles are situated close together near the anterior end.

The macronucleus presents no diagnostic characteristics, though, as the figure shows, in the culture under consideration it usually is proportionately larger in size than it is in the well-known species of *Paramecium*. Little emphasis, however, can

[22] A. Khainsky, "Zur Morphologie und Physiologie einiger Infusorien," *Arch. f. Protistenk.*, 21, 1910.

be placed on this as the size relations vary in all races of all species under different conditions. But I have never seen the macronucleus of *P. calkinsi* as small as Stokes figured it for *P. trichium*.

The micronuclear apparatus is almost identical with that of *P. aurelia* and in marked contrast with that of the other species (see Figs. 4 and 6). In the first place, there are characteristically

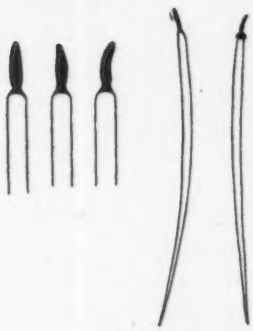

FIG. 5.

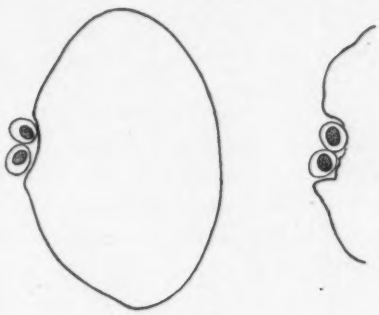

FIG. 6.

FIG. 5. 'Exploded' trichocysts of *Paramecium calkinsi*.

FIG. 6. Micronuclear apparatus of *Paramecium calkinsi*. Left, from a total preparation; right, from a section. Camera lucida sketches.

two micronuclei situated in or near a slight depression of the macronucleus. It has not been possible in every case to discover two micronuclei but there can be no doubt that two are typical of the vegetative cells. And, of course, as in all paramecia, the position of the micronuclei varies considerably in different individuals. Sometimes the macronuclear depression is on one side and sometimes on the other side. Frequently there are two tiny depressions each with a micronucleus. Again, the micronuclei may be at a considerable distance from the macronucleus, though not in mitosis.

In the second place, the relative size and the structure of the micronuclei are essentially similar to those of *P. aurelia*. The chromatin of the resting micronuclei is clumped to form an endosome which is surrounded by an 'achromatic' layer enclosed by a distinct nuclear membrane.

The general behavior as well as the 'avoiding reaction,' etc., of *P. calkinsi* is very similar to *P. caudatum* and *P. aurelia*, though the broad and slightly curved anterior end of *P. calkinsi* renders the revolutions on its long axis and its spiral path somewhat more conspicuous than in the more common species of *Paramecium*.

Paramecium calkinsi appears to be adapted to essentially the same environmental conditions as *P. aurelia* and *P. caudatum*. A series of experiments in which flasks of hay infusion were seeded with both *P. calkinsi* and *P. aurelia* showed that, as a rule, one and not both species attained a maximum development in the same flask. Usually within a few days there was a heavy growth of one and only a few struggling survivors, or none at all, of the other species. There was no indication that one was more hardy than the other under the conditions of the experiment; success or failure to dominate the culture apparently depended on which form 'got the start,' so to speak. It seems probable that the excretion products of the two forms are mutually detrimental and that this is the determining factor in the comparative incompatibility of the two species.[23] This point is being investigated.

The rate of reproduction of the new species, as exhibited in pedigree cultures throughout nearly a year, shows no factors which distinguish it from *P. aurelia*, *P. caudatum* or *P. bursaria*, which I have studied under similar conditions, since it is well known that characteristic division rates obtain in different races of the same species. The main pedigree culture of *P. calkinsi* was carried from January 30 to November 20, 1920, when it died out at the 321st generation (Fig. 7). During all this period a constant culture medium of standard beef extract was employed, and during certain experiments the temperature was maintained, by a thermostat, practically constant at 24° C. The race is now thriving in mass cultures which were seeded from the pedigree lines. The animals are apparently in as healthy condition as ever.

[23] L. L. Woodruff, "The Effect of Excretion Products of Infusoria on the Same and on Different Species, with Special Reference to the Protozoan Sequence in Infusions," *Jour. Exper. Zoöl.*, 14, 1913.

Throughout the year's work every effort has been made to secure conjugation and encystment in the mass cultures, though without result. Furthermore, an intensive study, involving the making of more than a thousand permanent preparations of pedigree animals at all stages in the life of the culture,[24] has failed to reveal any indications of endomixis. Therefore, it can be stated positively that endomixis has not occurred in this culture, although there are fluctuations of the division rate somewhat similar to those associated with endomixis in *P. aurelia* and *P. caudatum*.[25] This point will be considered in detail in another paper.

As is well known, the diet of *Didinium nasutum* is almost exclusively confined to paramecia, and it has not been possible to cultivate successfully the former without this food. An experiment extending through several months showed that *Didinium* thrives on *P. calkinsi* just as well as on *P. aurelia*—further evidence that the new species is a *Paramecium!*

From the foregoing description of the structure and life history of *P. calkinsi* it is apparent that this

[24] I am indebted to Miss Hope Spencer of the Osborn Laboratory for assistance in carrying out this work.

[25] Woodruff and Baitsell, "Rhythms in the Reproductive Activity of Infusoria," *Jour. Exper. Zoöl.*, 11, 1911. Woodruff and Erdmann, "A Normal Periodic Reorganization Process without Cell Fusion in *Paramecium*," *Jour. Exper. Zoöl.*, 17, 1914. Erdmann and Woodruff, "The Periodic Reorganization Process in *Paramecium caudatum*," *Jour. Exper. Zoöl.*, 21, 1916.

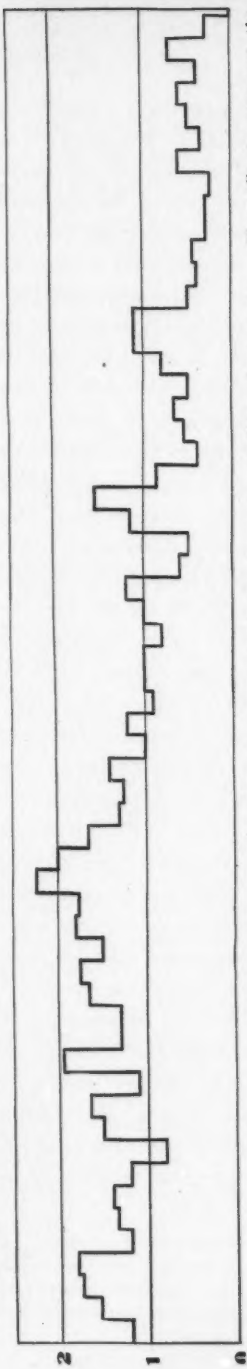

FIG. 7. Graph of the division rate of the main pedigree culture of *Paramecium calkinsi* from January 30 to November 20, 1920. The ordinates represent the average daily rate of division of the four lines of the culture, again averaged for five-day periods.

species presents a complex mosaic of the characters of the other members of the genus[26]—indeed the intrageneric relationships of the new species are its chief interest.

A survey of the six species of *Paramecium* under discussion shows that they fall into two quite clearly defined groups which may be referred to as the '*aurelia* group' and the '*bursaria* group' respectively. The members of the '*aurelia* group' (*P. aurelia*, *P. caudatum* and *P. multimicronucleata*) are characterized by a relatively long spindle- or cigar-shaped body; those of the '*bursaria* group' (*P. bursaria*, *P. putrinum*, *P. trichium* and *P. calkinsi*) by a somewhat shorter and broader form, with a tendency, especially prominent in *P. bursaria*, toward a dorsoventral flattening.

Within each of these groups are found two general structural types of micronuclei. One type, which may be termed the '*caudatum* type,' is exhibited by *P. caudatum* in the '*aurelia* group,' and by *P. bursaria*, *P. putrinum* and apparently by *P. trichium* in the '*bursaria* group.' Though Stokes' figure of the micronucleus of *P. trichium* leaves much to be desired, it clearly indicates that the single micronucleus is not of the 'endosome' type. The other type of micronucleus, the 'endosome' or '*aurelia* type,' occurs in *P. aurelia* and *P. multimicronucleata* in the '*aurelia* group,' and in *P. calkinsi* in the '*bursaria* group.' All species with the '*caudatum* type' characteristically possess one micronucleus, and those with the '*aurelia* type,' two micronuclei, except *P. multimicronucleata* in which the number apparently varies from six to nine.

Paramecium calkinsi represents the '*aurelia* type' of micronuclear apparatus in the '*bursaria* group' of species. It is possible that future study may show that certain of the paramecia under discussion (*e.g.*, *P. micronucleata*, *P. trichium*, *P. putrinum*) should be regarded as racial forms rather than distinct species, but I believe that the data presented above indicate beyond doubt that *Paramecium calkinsi* takes its place by *P. bursaria*, as *Paramecium aurelia* has by *P. caudatum*, as a distinct species.

[26] It is unnecessary to illustrate the cell form and micronuclear structure of the several species of *Paramecium* because figures are available in many of the monographs cited. Especial reference should be made to the figures given by Jennings and Hargitt, Schewiakoff, and Hamburger.

March 4, 1921.